



# THE JOURNAL

OF

# IOLOGICAL CHEMISTRY

BY CHRISTIAN A. HETTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HETTER MEMORIAL FUND

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VOLUME X

BALTIMORE

1911-12

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

WAVERLY PRESS  
BALTIMORE, MD

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## ANNOUNCEMENT

### THE CHRISTIAN A HERTER MEMORIAL FUND

The Directors of the JOURNAL OF BIOLOGICAL CHEMISTRY take this means of making known that the following friends and associates of the late Christian A Herter have contributed to a Memorial Fund in recognition of his labors in promoting medical science

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This fund, now amounting to forty thousand dollars, has been confided to the care of the Directors of the JOURNAL OF BIOLOGICAL CHEMISTRY under the provisions of a Declaration of Trust executed by them

The chief aim of the trust is to further the interests of the JOURNAL OF BIOLOGICAL CHEMISTRY, an instrument for the development of science created by Christian A Herter and fostered by him up to the time of his death. In the event that conditions arise removing the need for such a use of the income, provisions are made by which the fund shall continue as a memorial of Professor Herter and of service to humanity by the promotion of scientific research

A journal devoted to the interests of pure science is not likely to become a remunerative enterprise. As its usefulness enlarges the demands which it ought to meet tend also to expand. An appreciation of these facts has led to the formation of the memorial fund, which serves as a partial endowment of the Journal, giving it a financial support that insures its indefinite existence and assists materially in the development of the plans for its future, as formulated by Professor Herter

It has been no part of these plans that the Journal should accumulate profits beyond a reasonable reserve to meet exigencies, and the existence of this partial endowment would in any event make such a course improper. Should the finances in the future show a surplus in excess of these needs, it will be the policy of the Directors to apply this surplus in such ways as will increase and enlarge the usefulness of the Journal.

The immediate purpose of this announcement is to make known the existence of the Christian A. Herter Memorial Fund. But the Directors realize that the strength of the Journal lies only in part in its financial resources. The character of the Journal and its value depend chiefly upon the support of its contributors and subscribers, and the Directors take this opportunity to earnestly invite all who are interested in the advance of Biological Chemistry to cooperate with them in the endeavor to make the Journal representative of this branch of science in America.

SIMON FLEXNER, *President*  
A. N. RICHARDS, *Secretary*

# ON THE INSTABILITY OF GLUCOSE AT THE TEMPERATURE AND ALKALINITY OF THE BODY

(Preliminary Communication)

By LAWRENCE J HENDERSON

(From the Laboratory of Biological Chemistry of the Harvard Medical School)

(Received for publication, June 9, 1911)

All the monosaccharides are known to be very sensitive in a great variety of ways to the action of aqueous solutions of alkaline reaction. The chemical transformations—in the main rearrangements—which thus take place in sugar solutions under the influence of the hydroxyl ion group themselves into three classes

1 The processes underlying the phenomenon of mutarotation, which occur spontaneously, but are very greatly hastened by the presence of mere traces of hydroxyl ions<sup>1</sup>

2 The phenomenon of Lobry de Bruyn, gradual and nearly complete loss of optical activity accompanied by a partial transformation of a sugar into two (or more) other closely related sugars, which takes place with considerable velocity at moderate temperature in solutions of moderate alkalinity<sup>2</sup>

3 Complete destruction of the carbohydrate strong alkalies, acting for a long time or at high temperature, produce a very intricate tangle of simultaneous reactions which have not yet been adequately defined<sup>3</sup>

The oxidation of glucose is the most considerable chemical activity of the organism, and since the changes involved are known to take place at nearly constant alkaline reaction (almost neutrality) as well as at nearly constant temperature, it is natural

<sup>1</sup>See, for example, Hudson *Journal of the American Chemical Society*, **xxxii**, p 889, 1910

<sup>2</sup>See the papers of Lobry de Bruyn and Alberda van Eckenstein in *Recueil des travaux chimiques des Pays Bas*, **xiv-xix**

<sup>3</sup>See among recent publications, for example, Meisenheimer *Berichte*, **xli**, p 1009, 1908, Nef *Liebig's Annalen*, **ccclvii**, p 294, 1907, **ccclxxvi**, p 1, 1910

to inquire what may be the biological significance of the facts above enumerated. One further consideration, that the merest initiation of a reaction by the hydroxyl ions of protoplasm may serve as a key to unlock the sugar molecule so that an enzyme shall act upon it, lends weight to the problem.

The important studies of Sorensen,<sup>1</sup> among others, have shown the influence on the action of enzymes of slight variations in the concentration of hydrogen and hydroxyl ions. Such a possibility in the case of the enzymes which act on sugar has long been recognized in this laboratory, and, to minimize changes in alkalinity, a phosphate mixture was here employed by Hall<sup>2</sup> in his studies of glycolysis. In truth it is especially important to take into account changes in acidity and alkalinity in such cases, because the destruction of sugar is probably always accompanied by the production of acid, commonly in very large quantity.

Accordingly it seems probable that the very exact regulation of the alkalinity of the body has as one of its principal objects the preservation of a suitable medium for the destruction of glucose and the regulation of the process. To be sure Michaelis and Rona<sup>3</sup> have shown that under physiological conditions of temperature and alkalinity, and in the absence of enzymes, there is no considerable destruction of glucose in dilute solution in a few days. But such observations permit valid conclusions only for the purpose for which they were carried out—to show that investigations of glycolysis are not complicated by spontaneous destruction of sugar. On the other hand it is well established, by the same investigations of Michaelis and Rona among others, that at body temperature and very faint alkalinity solutions of glucose slowly lose their activity. The preliminary experiments reported in this paper now show that the same phenomenon occurs at body alkalinity and elevated temperature. Finally I have also found that at body temperature and alkalinity a very slow but quite unmistakable loss of optical activity does occur in glucose solutions.

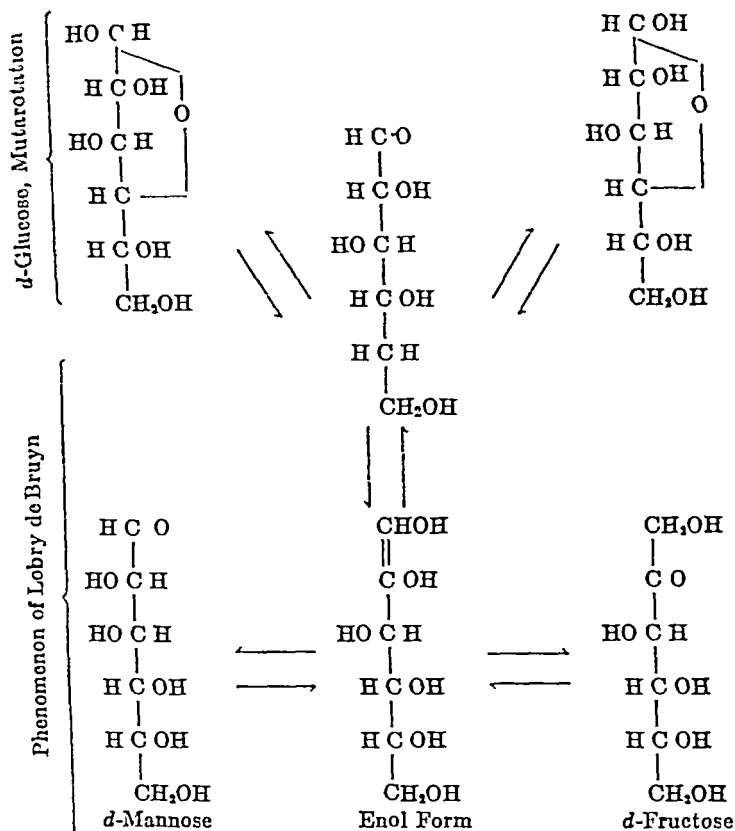
It is therefore necessary to suppose that the destruction of glucose at elevated temperature, or in solutions of more intense alkalinity reaction, is continuous with the process at body tem-

<sup>1</sup> *Comptes rendus des travaux du Laboratoire de Carlsberg*, viii, p. 1, 1909

<sup>2</sup> *Amer Journ of Physiol*, viii, p. 283, 1907

<sup>3</sup> *Biochem Zeitschr*, xiii, p. 364, 1910

perature and alkalinity<sup>1</sup> At all events, taking into account the facts of mutarotation and those established by Lobry de Bruyn and Alberda van Eckenstein, it is evident, in accordance with the present theoretical explanation of these phenomena, that in protoplasm glucose spontaneously undergoes the following reversible changes



<sup>1</sup> Unless indeed the phenomena here reported should be in part due to union of phosphoric acid with sugar, or some other specific chemical action between these two substances, as in alcoholic fermentation (Harden and Young) In any case these considerations suggest a possible explanation of the availability of the three sugars, glucose, fructose, and mannose for alcoholic fermentation and for the formation of the same compound with phosphoric acid from every one of them (Young *Biochem Zeitschr*, xxxii, p 177, 1911)



Such changes are due to the hydroxyl ion. It is clear that the unstable condition of the sugar, which finds its expression in these changes, may be the necessary preliminary to physiological oxidation. In that event, production of acid, for example in the muscle, should check the destruction of sugar, and thus the physiological process, like the spontaneous destruction of sugar by strong alkali, might be in a certain degree subject to a kind of auto-regulation.

## EXPERIMENTS

A series of solutions of glucose (Merck's highest purity) was prepared, each containing about 2.38 per cent of glucose, with a rotation, as measured in a two decimeter tube, of  $2.50^\circ$ . Each solution also contained a mixture of phosphates, in the ratio shown in the table, the concentration of phosphoric acid being about one-fifth molal. These solutions were boiled over free flames

TABLE I

SOLUTION	$\frac{\text{NaH}_2\text{PO}_4}{\text{Na HPO}_4}$	$(\text{OH}) \times 10^7$ 100°	ROTATION				
			0 HOUR	0.5 HOUR	1.5 HOURS	3.5 HOURS	24 HOURS
I	0.024	120	$2.50^\circ$	$0.48^\circ$			$-0.06^\circ$
II	0.062	50	$2.50^\circ$	$0.76^\circ$	$0.02^\circ$		$-0.08^\circ$
III	0.13	25	$2.50^\circ$	$1.10^\circ$	$0.19^\circ$		$-0.02^\circ$
IV	0.27	10	$2.50^\circ$	$1.54^\circ$	$0.54^\circ$		$+0.01^\circ$
V	0.60	5	$2.50^\circ$	$1.97^\circ$	$1.13^\circ$	$0.20^\circ$	Inactive
VI	1.6	2	$2.50^\circ$	$2.24^\circ$	$1.71^\circ$	$1.03^\circ$	Inactive

\* Rough approximations. In a neutral solution at 100° the concentration of ionized hydroxyl is not far from  $4 \times 10^{-7}$ .

with reflux condensers, and from time to time the optical activity of the solutions was measured.<sup>1</sup> The results are shown in Table I. Other monosaccharides seem to behave similarly, according to experiments which I have performed with arabinose, fructose, and galactose. Maltose, under such conditions, loses its rotatory

<sup>1</sup> It is a remarkable circumstance that this process leads to an almost or quite complete loss of optic activity. Especially is this true when the hydroxyl ion concentration is very nearly that at neutrality. It does not seem clear that a process like that described by Lobry de Bruyn should lead to complete loss of activity.

power very slowly. I am now trying to discover whether the process which leads to complete loss of optical activity is indeed a reversible reaction between several optically active sugars, or a true racemization. It is evident that slight optical activity at the end of the experiment may be due to the formation of relatively stable by-products, and the above experiments lend support to this view.

For studies of the process under physiological conditions three solutions of glucose (Merck's highest purity) were prepared, the concentrations being about 11.0 per cent, 12.6 per cent, and 5.4 per cent. Each solution also contained a phosphate mixture (approximately 9 parts  $\text{Na}_2\text{HPO}_4$  to 1 part  $\text{NaH}_2\text{PO}_4$ ) such that the concentration of phosphoric acid was approximately one-tenth molal. Each solution was divided into 8 fractions in small flasks, covered with toluol,<sup>1</sup> thoroughly shaken, stoppered, and

TABLE II

SOLUTION	ROTATION							
	April 3 2 days	April 5 4 days	April 7 6 days*	April 12 11 days	April 17 16 days	April 19 18 days	May 3 32 days	May 15 44 days
I	11 39°	11 29°	11 31°	11 23°	11 15°	11 11°	11 04°	10 90°
II	13 12°	13 06°	13 01°		12 86°	12 84°	12 72°	12 66°
III	5 56°				5 46°	5 43°	5 38°	5 31°
Total per cent loss of rotation		0.6	0.8	1.4	2.0	2.3	3.1	4.0

\* Average of 2 fractions. † Average of 4 fractions

carefully sealed with hard paraffine. These solutions were placed in a warm room at a temperature of  $38^\circ \pm 2^\circ$ . From time to time flasks were removed and the optical activity of the contents was measured.<sup>2</sup> There was never any indication of growth in the solutions, but the solutions gradually became faintly yellow. The results of the experiment are arranged in Table II.

The experiments here reported are being continued and extended.

<sup>1</sup> The toluol used for the first series of flasks contained 2 per cent of thymol.

<sup>2</sup> It is to be noted that the possibility of a reaction between sugar and phosphate has not been excluded in these experiments, nor is it certain that no formation of disaccharide takes place. See W. Löb *Biochem. Zeitschr.*, **XXII**, p. 43, 1911.



# A METHOD FOR THE STUDY OF PROTEOLYTIC FERMENTS

(Preliminary Communication)

By PHILIP ADOLPH KOBER

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(Received for publication, May 18, 1911)

The differentiation of the various ferments present in normal and pathological secretions is becoming a matter of considerable clinical importance. The use of peptides containing the tryptophane nucleus, which yield simple color reactions on hydrolysis has already proved to be of service in the diagnosis of gastric carcinoma<sup>1</sup>. The following preliminary note contains the results of experiments upon which it is hoped to base a useful method for following the action of ferments upon peptones and synthetic polypeptides.

The method proposed in this paper is based on the very characteristic behavior of the copper salts of amino-acids and polypeptides. Contrary to statements in scientific literature<sup>2</sup> the copper salts of amino-acids in alkaline solutions, particularly on warming or on boiling, precipitate copper as hydrate quantitatively. Peptones and peptides on the other hand give *very little* or *no hydrate*<sup>3</sup> under the same conditions. Essentially the method

<sup>1</sup> Neubauer and Fischer *Deutsches Archiv f. Klin. Med.*, xcvi, Heft 5-6, Lyle and Kober *N. Y. Medical Journal*, June 4, 1910

Abegg *Handbuch der anorganischen Chemie*, II, p. 534

<sup>2</sup> These facts enable us to test the purity of our polypeptides, as well as the completion of proteolysis by chemicals and ferments. Pure peptides that give a blue copper salt do not precipitate copper as hydrate with alkali. Complete hydrolysis is indicated when the copper salts of the digestive mixture precipitate all their copper with alkali.

consists in making copper salts of the neutral digestive mixtures, bringing them to boil and adding a small amount of alkali <sup>1</sup> If any amino-acids are present an immediate precipitate of copper hydrate occurs, which is directly proportional to the amount of amino-acids present, and if no precipitate is obtained amino-acids are absent

Thus far six peptides and six amino-acids have been tried and found adaptable to this technic Owing to difficulty in obtaining the necessary substances we have not yet been able to test the method more completely

Fischer, Abderhalden and their collaborators found that most of their peptides and amino-acids when boiled with freshly precipitated copper hydrate or copper carbonate formed soluble copper salts With the exception of those giving a biuret test with strong alkali, no information is given by Fischer and Abderhalden on the precipitability of the copper from these salts by alkali

Since most, probably all, peptides were tested for biuret reaction, it can be assumed that the precipitation of any copper hydrate found would have been recorded This indicates a general application of this copper hydrate method to all polypeptides

### *Qualitative Experiments*

For most of the polypeptides and amino-acids the qualitative method does not differ from the quantitative, in the case of glycylglycine the qualitative method is slightly modified to give quantitative results

To test for digestion of polypeptides, a portion of the neutralized or slightly alkaline digestive mixture is boiled for a few minutes with an excess of copper carbonate or freshly precipitated copper hydrate,<sup>2</sup> until copper salts have been formed of *all* the amino-acids and peptides in solution If the solution is not too dilute the time required for complete formation of copper salts is about fifteen minutes The excess of copper hydrate is then filtered off, the filtrate brought to boiling, and 5 to 10 cc of  $\frac{N}{10}$  alkali

<sup>1</sup> Fischer states that most polypeptides are stable to an excess of alkali (*Untersuchungen über Aminosäuren, Polypeptide und Proteine*, p 53, 1906)

<sup>2</sup> Copper oxide reacts too slowly for this purpose

added. If any amino-acids are present a precipitate of copper hydrate will form in the solution or on the sides and bottom of the beaker. If no precipitate<sup>1</sup> can be seen, particularly on boiling a few minutes, amino-acids are absent.

If any reduction occurs as is shown by the red cuprous precipitate, the solution is filtered free from reduced cuprous oxide, neutralized, and again treated with copper hydrate. Obviously, all reducing substances present should be oxidized in this manner before treating finally with copper hydrate. Small amounts of ammonia do not interfere, and larger amounts of ammonia can be

COPPER SALTS OF AMINO-ACID OR POLYPEPTIDE	RESULT ON BOILING WITH DILUTE ALKALI	
	Precipitate	Filtrate
Glycine	A large amount	Colorless
Alanine	A large amount	Colorless
Leucine	A large amount	Colorless
Aspartic acid	A large amount	Colorless
Asparagine	A large amount	Colorless
Tyrosine	A large amount	Colorless
Glycyl-glycine	None	Unchanged
Alanyl-glycine	None	Unchanged
Glycyl-aspartic acid	None	Unchanged
Alanyl-aspartic acid	None	Unchanged
Di-glycyl-glycine	None	Unchanged
Glycyl-tryptophane	None	Unchanged
Silk peptone	None	Unchanged
Witte's peptone	None	Biuret color reaction

boiled off, after the copper salts of the peptides and amino-acids had been formed. In the presence of peptones digestion of polypeptides can only be determined by the quantitative method.

The table given above indicates the results obtained by the application of the method to amino-acids and polypeptides.

The action of some ferments upon glycyl-glycine and alanyl-glycine has also been studied qualitatively. The ferments were

<sup>1</sup> A control should always be made on the medium and suitable corrections if any, should be made on the main result.

obtained from the following sources stool extract,<sup>1</sup> duodenal secretion, extract of intestinal mucous membrane, colon and typhoid bacilli. In each case the copper salts prepared from the mixtures after digestion were partly decomposed on boiling with alkali, indicating the formation of amino-acids.

### *Quantitative Experiments*

Experiments were made first of all to prove that freshly precipitated copper hydrate will react quantitatively with amino-acids. With this end in view portions of 0.0100 gm. of glycine were dissolved in 20 to 30 cc. of water and then boiled with excess of either copper carbonate or copper hydrate. The amount of copper oxide recovered from the filtrates on treatment with alkali is recorded in the following table.

NUMBER	AFTER ABOUT TWENTY MINUTES BOILING WITH CuCO <sub>3</sub> CuO RECOVERED	AFTER TWENTY TO THIRTY MINUTES BOILING WITH CuCO <sub>3</sub> CuO RECOVERED	AFTER BOILING TEN MINUTES WITH PRECIPITATED Cu(OH) <sub>2</sub> CuO RECOVERED
	gram	gram	gram
1	0.0031	0.0042	0.0053
2	0.0027	0.0044	0.0053
3	0.0033	0.0044	0.0047
4	0.0030	0.0045	0.0051
Average	0.0030	0.0044	0.0051
Glycine uncombined	0.0040	0.0020	0.0004

The last column shows results of considerable accuracy.

Glycyl-glycine when converted into its copper salt yields no copper hydrate when treated with alkali as described. In the presence of amino-acids the amount of copper hydrate obtained is too high but this error may be overcome by the use of alcohol. The following results were obtained when five to six volumes of alcohol were added to the copper salts before boiling with alkali.

<sup>1</sup> Containing a mixture of erepsin and trypsin.

GLYCYL-GLYCINE	GLYCINE	THEORETICAL AMOUNT CuO	CuO RECOVERED
0 1000	0 0100	0 0053	0 0058
0 1000	0 0100	0 0053	0 0054
0 1000	0 0100	0 0053	0 0056
Averages		0 0053	0 0056
0 1000	0 0050	0 0026	0 0027
0 1000	0 0050	0 0026	0 0025
Averages		0 0026	0 0026

The following results were obtained with alanyl-glycine and with glycyl-tryptophane, using the simple method described for qualitative work

POLYPEPTIDE	AMINO-ACID	THEORETICAL AMOUNT CuO	CuO RECOVERED
<i>gram alanyl-glycine</i>	<i>gram glycine</i>		
0 10	0 010	0 0053	0 0049
0 10	0 010	0 0053	0 0047
0 10	0 010	0 0053	0 0055
Averages		0 0053	0 0050
<i>grams glycyl-tryptophane</i>	<i>gram glycine<sup>1</sup></i>		
0 0815	0 010	0 0053	0 0045
0 0815	0 010	0 0053	0 0044
Averages		0 0053	0 0044

<sup>1</sup>A small amount of dextrose was present

The low results with glycyl-tryptophane were due to the use of copper carbonate in place of copper hydrate

It is proposed to test the method upon other polypeptides and also to follow the course of ferment action under various conditions. The study of the proteolysis of synthetic polypeptides is very difficult in the presence of other peptones but experiments are being made to devise a suitable method



## SUMMARY

Most amino-acids, if not all, form copper salts that yield their copper as hydrate when boiled with a slight excess of alkali

Most of the polypeptides that form copper salts do not yield their copper as hydrate when boiled with a slight excess of alkali

Peptones, which are probably mixtures of polypeptides, form copper salts that do not yield their copper as hydrate when boiled with a slight excess of alkali. Peptones dissolve more copper hydrate in the presence of alkali than in its absence

It is possible to base a method for the study of ferment action upon the foregoing facts

My thanks are due to Drs Marshall and Hager for assistance in the preparation of the polypeptides, to Mr Sugura for much routine work and to Drs Lyle and Parry for applying the method to the detection of ferments

# THE ANALYSIS OF PROTEINS BY DETERMINATION OF THE CHEMICAL GROUPS CHARACTERISTIC OF THE DIFFERENT AMINO-ACIDS

BY DONALD D VAN SLYKE

(From the Laboratories of the Rockefeller Institute for Medical Research, New York )

(Received for publication, June 17, 1911 )

The methods for isolating the amino-acids yielded by hydrolyzed proteins, as introduced by Kossel and Fischer, and further developed by other workers, have given us most of our present knowledge of the composition of proteins, and still constitute the means of attaining the most complete information concerning their chemical nature. These methods are still encumbered by two inherent difficulties, however, which limit their application in problems involving the chemical nature of proteins. (1) A large supply of material is required, 20 or 30 grams of protein for the determination of the bases by Kossel's method, while at least 100 grams, and preferably 300 or 400 grams, are used for the esterification. (2) The methods for determining most of the amino-acids are not quantitative. As a consequence, the most careful work leaves from a third to a half of the protein molecule still unaccounted for.

The analysis outlined below<sup>1</sup> is designed to enable one to attain an insight into the composition of proteins by methods which require but small amounts of material and yield approximately quantitative results, indicating the nature of all the nitrogenous products yielded by complete acid hydrolysis.

The analysis is based, not on the isolation of the amino-acids, but on determinations of their characteristic *chemical groups*.

<sup>1</sup> A brief account of the method as developed a year ago was published in the *Ber d d chem Ges*, 1910 p 3176. The method here published is different in many details from its previous form, which it supersedes.

By precipitation with phosphotungstic acid the amino-acids are separated into two fractions, the "bases," which are precipitated, and the other acids, which are not. The proportions in which the different types of amino-acids are present in each fraction are then ascertained by determination of their characteristic chemical groups.

Phosphotungstic acid as a precipitant for the basic amino-acids obtained by hydrolysis of proteins was introduced by Drechsel,<sup>1</sup> who with its aid discovered *lysine*. Soon afterwards Hedin<sup>2</sup> discovered arginine among the bases precipitated by phosphotungstic acid, and then histidine.<sup>3</sup> Kossel and Kutscher later devised the well known method for determining lysine, histidine, and arginine.<sup>4</sup> It was observed that the phosphotungstic precipitate constituting the "lysine fraction" sometimes contained a nitrogenous substance other than lysine and Winterstein identified this as *cystine*.<sup>5</sup> The large amount of work done on proteolytic products has discovered no amino-acids, other than the above four, which are present after complete acid hydrolysis of proteins and are precipitated in *dilute* solution by phosphotungstic acid. Positive evidence that the phosphotungstates of these amino-acids constitute the entire precipitate was afforded by Osborne and his co-workers. Osborne and Harris<sup>6</sup> ascertained the conditions necessary for determining quantitatively the proportion of "basic nitrogen," or that precipitated by phosphotungstic acid in dilute solutions of completely hydrolyzed proteins. Using the method of Osborne and Harris, Osborne, Leavenworth, and Brautlecht<sup>7</sup> found that, in a large series of proteins analyzed, the percentage of nitrogen precipitated by phosphotungstic acid agreed within reasonably close limits with that of the histidine, arginine, and lysine, as determined by Kossel's method. That these authors overlooked the cystine could easily occur from the fact that, as will be shown later, a large proportion of it is altered by the pro-

<sup>1</sup> *Arch f Anat u Physiol*, 1896, p 254

<sup>2</sup> *Zeitschr f physiol Chem*, *xx*, p 186, 1895

<sup>3</sup> *Ibid*, *xxii*, p 191, 1896

<sup>4</sup> *Ibid*, *xxxi*, p 165

<sup>5</sup> *Ibid*, *xxxiv*, p 153

<sup>6</sup> *Journ Amer Chem Soc*, *xxv*, p 323, 1903

<sup>7</sup> *Amer Journ of Physiol*, *xxiii*, p 194, 1908

longed boiling with acid necessary to completely hydrolyze some proteins. Consequently, although cystine, is readily precipitated, the precipitate actually obtained from proteins contains half or less of the original cystine nitrogen.

The work above cited indicates that arginine, histidine, and lysine, may be separated by means of phosphotungstic acid with approximately quantitative completeness from the other amino-acids. This has been confirmed by our experience, the details of which appear later in this paper, and which show that the precipitation of the unaltered cystine is also almost complete.

The principle of the scheme of analysis is briefly outlined in the following paragraph.

#### PRINCIPLE OF THE METHOD

After the removal of the ammonia by vacuum distillation, the arginine, histidine, lysine, and cystine are precipitated with phosphotungstic acid. The precipitate is redissolved, and these four "bases" are determined by utilization of their marked chemical differences. The determination of the amino nitrogen of this fraction at once divides it into pairs, lysine, and cystine, containing only amino nitrogen, and arginine and histidine, containing respectively three-fourths and two-thirds of their nitrogen in other forms<sup>1</sup>. Of the first pair, the cystine is given by the sulfur content, the lysine by subtracting the cystine from the sum of the two. Of the second pair, the arginine is determined by decomposing it with alkali, which drives off half its nitrogen as ammonia, and the histidine is obtained by difference. In the filtrate from the bases the amino-acids are divided into two sub-fractions. (1) The acids containing only primary amino nitrogen (leucine, alanine, etc.), (2) Those containing nitrogen in pyrrolidine (proline, oxyproline) or indol (tryptophane) rings.

In order that this method of analysis may be applied, it is, of course, necessary that the protein shall be free from nitrogenous impurities, such as purine bases.

The scheme of analysis is represented diagrammatically by the table on page 19. That the sub-fraction of the filtrate, con-

<sup>1</sup> Van Slyke, *This Journal*, 17, p. 485, 1911

taining 100 per cent of amino nitrogen may contain some acids at present unknown is possible, because the chief losses in hydrolyses by the methods of isolation fall here. As, however, the ester method, by which all of these acids except glutaminic and tyrosine must be determined, is at present accompanied by unavoidable losses, the discrepancies may well be due to these losses, rather than to the presence of unknown  $\alpha$ -amino-acids.

#### METHODS IN DETAIL

*Hydrolysis* A successful hydrolysis has been performed, using only 1 gram of protein (see hemoglobin hydrolysis), but for most satisfactory conditions 3 grams are required, and, if material suffices, it is well, as in all analyses, to perform duplicates, using 6 grams. The protein is dissolved in 10 or 20 parts of 20 per cent hydrochloric acid, and boiled in a tared flask under a reflux. At intervals of six or eight hours the hydrolysis is stopped, the solution cooled, and portions of 1.00 or 2.00 cc (enough to contain about 0.1 gram of protein) are withdrawn by a pipette. These portions are diluted to 10 cc and used for determination of the amino nitrogen. The different determinations of the series should all be run under the same conditions, as otherwise the ammonia from the amid nitrogen might cause an error in the determination. Ordinarily it is most satisfactory to run all the determination 6 minutes, the solutions of amino-acids and nitrous acid being mixed and allowed to stand for five minutes, then shaken for one minute. Under these conditions, room temperature being fairly constant, the same proportion of the ammonia (15-20 per cent at 20°) is decomposed in each case. After removal of each portion for amino determination, the flask in which the hydrolysis is performed is weighed, and the weight is again taken, after the hydrolysis has been continued 6 to 8 hours longer, before the next sample is withdrawn. These weights serve to detect concentration of the solution by loss of vapor. In case such concentration occurs, a correction for the percentage of volume decrease of the solution is made. The hydrolysis is continued until the amino nitrogen becomes constant. This shows when the hydrolysis is complete, and enables one to avoid the errors, which as Osborne has shown, may result from incomplete hydrolysis. (For an example

TABLE I

Precipitated by phosphotungstic acid	100 per cent of N as $\text{NH}_2$	(S) Cystine	$\begin{array}{c} \text{S}-\text{CH}-\text{CH}-\text{NH}_2-\text{COOH} \\   \\ \text{S}-\text{CH}-\text{CH}-\text{NH}_2-\text{COOH} \end{array}$
		(No S) Lysine	$\text{NH}_2-(\text{CH}_2)_4-\text{CH}-\text{NH}-\text{COOH}$
	Non-Amino Nitrogen	(Guanidine group) Arginine	$\begin{array}{c} * \\ \text{NH}=\text{C}(\text{NH}_2)-\text{NH} \\   \\ (\text{CH}_2)_3-\text{CH}- \\   \\ (\text{NH})\text{COOH} \end{array}$
		(No guanidine group) Histidine	$\begin{array}{c} * \quad \text{CH} \quad * \\ \text{NH} \quad \diagup \quad \diagdown \quad \text{N} \\   \quad \quad \quad   \\ \text{CH}=\text{C}-\text{CH}- \\   \quad \quad \quad   \\ \text{CH}(\text{NH})-\text{COOH} \end{array}$
Not precipitated by phosphotungstic acid	100 per cent of N as $\text{NH}_2$	Glutaminic acid	$\text{HOOC}-\text{CH}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$
		Aspartic acid	$\text{HOOC}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$
		Tyrosine	$\text{OH}-\text{C}_6\text{H}_4-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$
		Phenyl alanine	$\text{C}_6\text{H}_5-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$
		Serine	$\text{OH}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$
		Leucine	$\begin{array}{c} \text{CH}_3 \\ \text{CH}_3 \end{array} > \text{CH}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$
		Isoleucine	$\begin{array}{c} \text{CH}_3 \\ \text{C}_2\text{H}_5 \end{array} > \text{CH}-\text{CH}(\text{NH}_2)-\text{COOH}$
		Valine	$\begin{array}{c} \text{CH}_3 \\ \text{CH}_3 \end{array} > \text{CH}-\text{CH}(\text{NH}_2)-\text{COOH}$
		Alanine	$\text{CH}_3-\text{CH}(\text{NH}_2)-\text{COOH}$
		Glycocoll	$\text{CH}_2(\text{NH}_2)-\text{COOH}$
	Non-Amino N	Proline	$\begin{array}{c} \text{CH}_2 \quad \text{CH}_2 \\   \quad \quad   \\ \text{CH}-\text{NH}-\text{CH}-\text{COOH} \\   \quad \quad   \\ \text{OH}-\text{CH}_2 \quad \text{CH}_2 \end{array}$
		Oxyproline	$\begin{array}{c} \text{CH}_2 \quad \text{CH}_2 \\   \quad \quad   \\ \text{CH}-\text{NH}-\text{CH}-\text{COOH} \\   \quad \quad   \\ \text{CH}_2 \quad \text{CH}_2 \end{array}$
		Tryptophane	$\text{C}_6\text{H}_5-\text{N}^+ \text{CH}(\text{NH}_2)-\text{COOH}$

\*Non-amino nitrogen atoms (not reacting with nitrous acid This includes the  $\text{NH}_2$  group of the guanidin nucleus in arginin)

of this method of following the hydrolysis, see the gliadin analysis described in the latter portion of this paper )

*Determination of Ammonia (Amid Nitrogen)* The determination of ammonia yielded by hydrolyzed proteins has become especially significant since Osborne, Leavenworth, and Brautlecht have shown that the nitrogen of the ammonia is usually equal to that of the dicarboxylic acids, with which it is presumably combined in the protein molecule in the form of acid-amid radicles

In order that the subsequent determinations may not be interfered with, it is necessary that every trace of ammonia be removed when it is determined, but that the treatment with alkali be so mild that neither the arginine nor the cystine is attacked Denis<sup>1</sup> has shown that boiling at 100° with even so weak an alkali as magnesium oxide attacks cystine and drives off a portion of its nitrogen as ammonia We have noted the same behavior of cystine, although arginine is not attacked As a result of the sensitiveness of cystine one must drive off the ammonia at room temperature, using either the aeration method of Denis, or vacuum distillation After trying various modifications of both principles, the following technique was adopted as the most convenient and certain

The solution of hydrolyzed protein is placed in a small double-necked distilling flask, and concentrated under diminished pressure until all the hydrochloric acid possible has been driven off The residue is taken up with warm water, and the solution is transferred to a measuring flask of 100 to 250 cc capacity, according to the amount of protein hydrolyzed Aliquot portions of sufficient size to contain about 0.2 gram of protein are withdrawn and used for Kjeldahl analyses, which give the total nitrogen, on the basis of which the other determinations are calculated

For the determination of the ammonia, distillation under diminished pressure with lime is used No special apparatus is required, a one-liter, double-necked distilling flask, an ordinary one-liter distilling flask, and a 200 cc distilling flask being arranged as shown in Fig 1 As indicator in the  $\frac{N}{16}$  acid, alizarine sulfonate is used The solution, containing about 3 grams of hydrolyzed protein, is placed in the double-necked flask, and diluted to about

<sup>1</sup> This *Journal*, VIII, p 365

200 cc One hundred cubic centimeters of alcohol, to prevent foaming during distillation, is added, then a 10 per cent suspension of calcium hydrate until a slight excess is present, as shown by the turbidity and alkaline reaction of the solution. The apparatus is then joined together as shown in the figure and evacuated to a pressure of 30 mm or less. The Claissen flask is then placed in a bath at 45°–50°, and the solution distilled for a half-hour. In case distillation starts too rapidly, a little air is let in from the stop-cock in one neck of the Claissen flask. When the distillation is finished the flask is lifted from the water bath, and the vacuum is released by opening this stop-cock. The  $\frac{N}{16}$  acid from the receiving flask and the

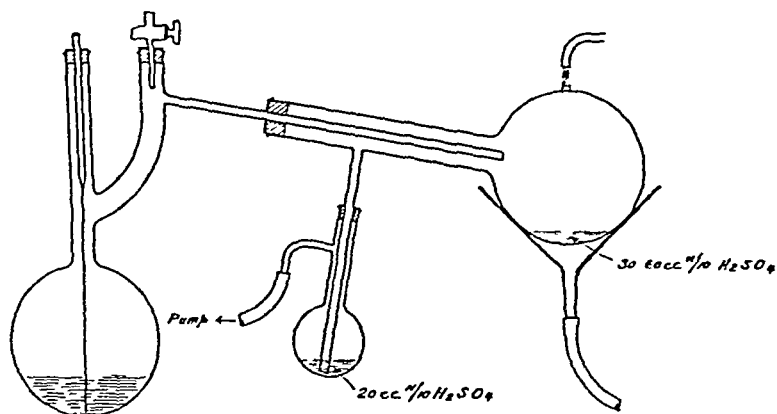


Fig 1 Apparatus for determining amid nitrogen by vacuum distillation

smaller guard flask is washed into a half liter beaker or Erlenmeyer and titrated back with  $\frac{N}{16}$  NaOH. The amount of  $\frac{N}{16}$  acid in the larger flask is usually 30 cc when an animal protein is being analyzed, 60 cc when the protein is of plant origin, as some plant proteins yield much ammonia.

*Melanin Nitrogen* During the distillation all of the black coloring matter, or melanin, which is formed during the hydrolysis of proteins, is adsorbed by the undissolved lime. The latter is filtered off on a folded filter and washed with water until the washings come free of chloride. The precipitate and paper are then submitted to Kjeldahl analysis, using 35 cc of sulfuric acid to



digest the large amount of organic matter in the paper. In this determination, as in the ammonia distillation, the lime performs the function of the magnesia in the nitrogen distribution method of Osborne and Harris<sup>1</sup>

*Precipitation, Washing, and Redissolving of the Bases* The filtrate from the melanin is neutralized with hydrochloric acid, returned to the vacuum distilling flask, and concentrated to about 100 cc. It is then washed into a 200 cc. Erlenmeyer, and 18 cc. of concentrated hydrochloric acid and a solution containing 15 grams of phosphotungstic acid are added. The entire solution is then diluted with water up to 200 cc. and heated in a water bath until the precipitate of the bases is nearly or quite redissolved. The bases reprecipitate on cooling as crystalline or granular phosphotungstates which can be readily washed and filtered. The above conditions of precipitation are practically those of Osborne and Harris, with the exception that, in order to avoid precipitation of calcium sulfate, hydrochloric acid is used here instead of sulfuric. The solution is allowed to stand forty-eight hours for the precipitate to form, for in less time the precipitation of histidine may be incomplete.

The *washing* of the phosphotungstic precipitate has been one of the most troublesome points of the "nitrogen distribution" method of Hausmann. Against this method (the division of the amino-acids into the fraction precipitated by phosphotungstic acid and the fraction not precipitated) Kossel raised the objection that the results varied greatly according to the manner in which the precipitate was washed. Osborne and Harris, however, showed that when a moderate volume of a solution containing 2.5 per cent of phosphotungstic acid and 5 per cent of sulfuric was used to wash the precipitate the results were constant. These authors allowed the precipitate to drain on the filter, then suspended it in the washing solution and allowed it to drain again, washing it three times by this method. The slight amount of nitrogen in the mother liquors not washed out of the precipitate was approximately balanced by the slight amount of the bases dissolved in the mother liquors and washings.

For our purpose, however, it is necessary that the precipitate

<sup>1</sup> *Journ. Amer. Chem. Soc.*, xlv, p. 323

shall be washed entirely free of the mother liquors and the amino-acids of the unprecipitated fraction. It is also necessary that as small an amount of the washing solution as possible shall be used, in order that the precipitate, which is slightly soluble in the washing solution, shall not dissolve to an appreciable extent during the washing. These requirements are met by the following technique, which permits the quantitative washing of the precipitate with 100-200 cc of solution, and without dissolving appreciable amounts. A *hardened* filter paper is cut to the proper size to fit accurately against both the bottom and side walls of a 3-inch Buchner funnel. The part of the filter paper which lies against the sidewalls of the funnel is folded in about twenty plait, so that it fits the wall snugly all the way around. Into the pocket thus formed by the filter paper the precipitate is poured, and the mother liquors are drawn off as completely as possible by suction, the precipitate being pressed down by a flattened rod. The filtrate is transferred from the suction flask to a beaker. Onto the precipitate in the funnel are poured 10-12 cc of a washing solution containing 2.5 per cent phosphotungstic acid and 3.5 per cent of hydrochloric acid, and the precipitate and solution are stirred up together until a smooth suspension is formed. Care must be taken that all the lumps are broken up, and the precipitate completely reduced to a granular suspension. It is then sucked dry, as before. The washing in this manner is repeated until the filtrate comes free of calcium, from eight to fifteen washings being required, according to the bulk of the precipitate. The first three or four portions of washing solution are used to dislodge the last granules of the precipitate from the flask in which the latter formed. The succeeding portions are added from a wash bottle or a pipette in a fine stream around the edge of the filter paper, so that the latter is washed from the edge downwards around its entire circumference. In case the first four washings leave a few granules of the precipitate still in the flask, they are allowed to remain there, as they are already sufficiently washed, and the subsequent portions of the washing solution are used to wash the filter paper as well as the precipitate, in the manner just described. In case the later washings run through somewhat turbid, as is often the case, they are filtered through a small folded filter before being combined with the main filtrate. The phosphotungstic acid used in the

precipitation and in the washing solution is purified by shaking with ether and water after the method of Winterstein

In testing the washings for calcium, a solution of oxalic acid in 3 per cent sodium hydrate is used. To about 1 cc of this solution one adds two or three drops of the filtrate and shakes slightly until the upper layer, where the filtrate remains, becomes alkaline. The washings are continued until a sample of the filtrate gives no trace of turbidity after standing several minutes with the oxalate solution.

The washing being finished, the precipitate is removed as completely as possible by means of a spatula and a wash bottle with distilled water, to a beaker holding more than a liter. When the precipitate has been removed as completely as possible by mechanical means, the filter paper is spread out on the bottom of a dish and washed with water made alkaline by addition of a few drops of 20 per cent sodium hydrate. This dissolves the portions of precipitate imbedded in the fibers of the filter paper. The small folded filter paper used for filtration of the later washings is similarly freed of adherent precipitate. Also, in case any granules of the precipitate have remained in the flask in which the precipitate originally formed, these are either washed or dissolved out, and added to the other portions in the large beaker. To the suspension therein one adds a few drops of phenolphthalein solution, then 50 per cent NaOH solution drop by drop, with stirring. As soon as the solution becomes red, addition of the alkali is discontinued until the color fades again. All of the precipitate is soon brought into solution in this manner. The solution must be red at the end, but must not contain more than three or four drops of alkali in excess of the amount required to neutralize it. Greater excess of alkali is to be avoided because of the sensitiveness to it of cystine and arginine.

The solution is diluted to about 800 cc, and a 20 per cent solution of crystalline barium chloride is added in portions of a few cubic centimeters each, until a test with neutral sodium sulphate solution shows the presence of an excess of barium above the amount required to precipitate the phosphotungstic acid. In case the solution loses its red color during the precipitation, two or three drops more of the alkali solution are added, as the precipitation is not satisfactory unless the solution is slightly alkaline.

The barium solution must be added until the test results in an immediate granular precipitate of barium sulfate. A detectible turbidity can be obtained before enough barium chloride has been added to complete the precipitation of the phosphotungstate. On the other hand, more barium chloride than a few cubic centimeters excess is to be avoided, as a large excess of barium causes disagreeable bumping when the solution is boiled later in the arginine determination. The dilution which is directed before precipitation is necessary in order to avoid appreciable loss of the bases through adsorption by the barium phosphotungstate.

The precipitate of barium phosphotungstate is filtered and washed with water, the filtration and washing being performed in the manner described for the phosphotungstates of the bases, except that such small portions of wash water do not need to be used. The same funnel and piece of hardened filter paper can usually be used to advantage for both filtrations. The washing is continued until the filtrate comes free of chloride. The filtrate is concentrated *in vacuo* in the same double-necked distilling flask used for the determination of amid nitrogen, the concentration being continued until the volume of the solution is reduced to about 50 cc. During the concentration, a small amount of barium phosphotungstate which was not precipitated at first separates from the solution. The latter is filtered into a 200 cc double-necked distilling flask, the filter being washed with water until chloride free. The solution is then concentrated, and transferred to a 50 cc measuring flask.

*Determination of Arginine* The determination of arginine is based on the fact, first noted by Osborne, Leavenworth, and Brautlecht, that arginine, when boiled with dilute alkali evolves half of its nitrogen in the form of ammonia. The explanation of the reaction is that, as shown by Schulze and Winterstein, arginine when heated with alkaline solutions decomposes into one molecule each of urea and ornithine. The urea then is decomposed into ammonia. Under the conditions described below the reaction is quantitative. Of the 50 cc of solution containing the bases, 25 cc are placed in the 200 cc Jena Kjeldahl flask of the apparatus shown in Fig 2. The Folin bulbs at the top of the condenser of the apparatus are connected with the condensing tube by a ground glass joint, as shown in the figure, or by a very heavy

piece of rubber tubing, which is less convenient but equally efficient. In the Folin tubes one places 15 cc of  $\frac{N}{10}$  acid, with alizarin sulfonate as indicator. To the solution in the flask one adds 12.5 grams of solid KOH, and a bit of porous porcelain to prevent bumping. The solution is then boiled gently for exactly 6 hours. At the end of this time the Folin tube is disconnected from the

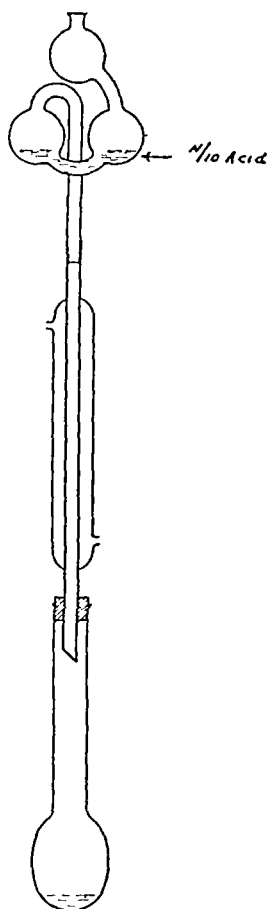


Fig 2 Apparatus for determination of arginine

condenser, and through the latter 100 cc of water are poured into the Kjeldahl flask. The solution in the flask contains a slight amount of ammonia, although the greater part diffuses up into the Folin bulbs during the 6 hours boiling, and is absorbed by the acid. In order to obtain also the small amount remaining in the

flask, the latter is connected with the condenser of an ordinary Kjeldahl still and the ammonia driven off in the customary manner. The receiver contains the acid from the Folin bulbs, so that all the ammonia from the arginine determination is collected in one solution of  $\frac{N}{10}$  acid. Care must be taken that not more than 100 cc of water is boiled off during the distillation, for if the alkaline solution becomes too concentrated other decompositions besides the desired one will result. The excess of  $\frac{N}{10}$  acid in the receiver is titrated back as usual. Each cubic centimeter neutralized by the ammonia indicates 0.0028 gram of arginine nitrogen in the solution decomposed, or 0.0056 gram in the total solution of the bases. In case cystine is present, 18 per cent of its nitrogen is evolved as ammonia during the arginine determination, and a corresponding correction to the arginine figures must be made. The correction is, however, practically negligible with all ordinary proteins except the keratins. As the behavior of the cystine under the conditions of the determination is quite constant, the accuracy of the arginine determination is not seriously affected even in analysis of the keratins. The cystine determination, from which the correction can be accurately made, is described below.

The 200 cc Kjeldahl flasks should not be used for more than two or three arginine determinations, as the glass is attacked by the strong alkali. Unfortunately, copper flasks cannot be used because of the effect of the copper in oxidizing the cystine present.

*Determination of the Total Nitrogen of the Bases* The solution used for the arginine determination is transferred from the 200 cc Kjeldahl flask to one of 500 cc. Thirty-five cubic centimeters of concentrated sulfuric acid are cautiously added, with cooling, and 0.25 gram of copper sulfate. The solution is then digested as in an ordinary Kjeldahl determination, and its nitrogen content determined. The cubic centimeters of  $\frac{N}{10}$  acid neutralized in this determination are added to those neutralized in the arginine determination. The sum multiplied by 0.0028 gives the total nitrogen content of the bases. The use of the same portion of solution for the determinations of both arginine and total nitrogen of the bases permits one to use half the solution for them, and obtain each more accurately than would be possible if separate and smaller portions were used for each determination.

*Determination of Cystine* The amount of cystine present with the bases is obtained from the content of the solution in organic sulfur. For this determination the most accurate and convenient method is that based on Benedict's principle of oxidation by ignition with copper nitrate<sup>1</sup>. We have used the modification of Denis<sup>2</sup>. The oxidation is invariably complete, not a trace of carbon or other insoluble matter remaining. We have found, consequently, that one can safely proceed with the sulfur determination without first removing the barium chloride present in the solution of the bases. Ten cubic centimeters of the solution are placed in a porcelain evaporating dish of 7 to 10 cm diameter with 5 cc of Denis' solution. The mixture is concentrated to dryness on the water bath, then gradually heated to redness and maintained at that temperature for some minutes, as directed by Benedict. The residue is dissolved in 10 cc of 10 per cent hydrochloric acid, and the solution diluted to about 150 cc. It is heated to boiling, and, in order to make sure that an excess of barium chloride is present, 10 cc of a 5 per cent solution are added. The barium sulphate is washed and weighed as usual. Each milligram of barium sulfate indicates 0.06 mg of cystine nitrogen in the portion of solution analyzed, or 0.3 mg in the total solution of the bases. The weight of barium sulfate obtained must be corrected for the amount of sulfur found in the reagents by blank analyses. In the reagents used by us the correction was 1.5 mgs of barium sulfate. Reagents requiring a much larger correction should not be used, as the cystine present often yields only a few milligrams of barium sulphate.

The cystine actually present in the solution of the bases can be very accurately determined by the above method, for an error of 1.5 mg in the barium sulphate weighed, which is as large a margin as duplicates usually differ by, introduces an error of only 0.1 per cent in the total percentage of cystine nitrogen calculated. As about half the cystine originally present is altered during the hydrolysis with acids, however, and an amount containing 0.5 per cent of the nitrogen of the protein remains in solution when the bases are precipitated, the amount of cystine obtained by the above method

<sup>1</sup> This *Journal*, vi, p. 363, 1909

<sup>2</sup> *Ibid*, viii, p. 401

represents less than half that actually present in the protein. The precipitate of the bases from proteins not unusually rich in cystine contains, therefore, but a small proportion of its nitrogen in the form of cystine. This explains why the presence of cystine in the phosphotungstate precipitate has been overlooked by most workers, with the exception of Winterstein<sup>1</sup>

*Determination of Amino Nitrogen of the Bases* Ten cubic centimeters of the solution are used for this determination, which is performed in the usual manner. Because of relative slowness with which the  $\omega$ -amino group of lysine reacts, the determination must be continued for a half-hour at 20°, or for a somewhat longer time if the temperature is lower. A blank determination of the reagents must be run for the same length of time. Cystine gives off gas equivalent to 107 per cent of the amount of nitrogen which it should yield, and therefore a corresponding correction for the cystine is to be made to the amino determination. Except in proteins like the keratins, which are unusually rich in cystine, this correction is negligible, however.

*Calculation of Histidine* The non-amino nitrogen of the bases comes from the arginine, which contains three-fourths of its nitrogen in a form which does not react with nitrous acid, and from the histidine, which contains two-thirds of its nitrogen in non-amino form. Therefore to calculate the histidine nitrogen, we subtract three-fourths of the arginine nitrogen from the total non-amino nitrogen, and multiply the difference by  $\frac{3}{2}$ .

Or, letting  $D$  represent the total non-amino nitrogen of the bases (difference between total nitrogen and amino nitrogen), and  $Arg$  represent the arginine nitrogen, determined as previously described, we have the formula

$$\begin{aligned}\text{Histidine N} &= \frac{3}{2} (D - \frac{3}{4} Arg) \\ &= 1.667D - 1.125 Arg\end{aligned}$$

The histidine figure is more liable to error than that of any of the other three amino-acids of the basic fraction, because it is affected by error in the determination of either arginine total nitrogen, or amino nitrogen. In these determinations errors of  $\pm 1$  per cent would cause errors of  $-1.125$ ,  $+1.5$ , and  $-1.5$  per cents

<sup>1</sup> *Loc cit*



respectively in the histidine nitrogen. As, however, they can all be performed accurately, there is no reason why fairly constant results for the histidine should not be obtained. As a matter of fact our duplicates for histidine usually agree within a per cent.

*Calculation of Lysine* The nitrogen of the other three amino-acids of this fraction having been calculated, the lysine is obtained by difference. Or

$$\text{Lysine N} = \text{Total N} - (\text{Arginine N} + \text{Cystine N} + \text{Histidine N})$$

At first sight it would appear that the lysine determination, since it involves the figures for all the other three acids of the fraction, should be more subject to inaccuracy than the histidine determination. This is not the case, however. The accuracy of the lysine calculation depends chiefly upon that of the determinations of cystine and the amino nitrogen of the fraction, which are usually very accurate. An error of  $\pm 1$  per cent in any of the four determinations made on the bases would cause an error in the lysine nitrogen indicated by one of the following figures: amino nitrogen,  $\pm 1.5$  per cent, cystine nitrogen,  $-1$  per cent, total nitrogen,  $-\frac{1}{2}$  per cent, arginine nitrogen,  $+\frac{1}{3}$  per cent.

*Determination of the Total Nitrogen in the Filtrate from the Bases* To the combined filtrate and washings from the phosphotungstate precipitate of the bases 50 per cent sodium hydrate solution is added until the solution turns slightly turbid by precipitation of lime. It is then cleared again by addition of a little acetic acid. In adding the alkali, it is essential that the neutral point should not be passed by more than a few drops, as otherwise a precipitate may form which will not redissolve. The solution is placed in a double-necked distilling flask and concentrated under diminished pressure until salt begins to crystallize. The solution is then washed into a 150 cc measuring flask and diluted up to the mark. Duplicate portions of 25 cc each are taken for Kjeldahl determinations. For each portion one uses 15 grams of potassium sulfate, 35 cc of concentrated sulfuric acid, and 0.25 gram of copper sulfate. The sulfuric acid must be added carefully and under a hood, because of the vigorous evolution of hydrochloric acid gas. The digestion is continued for three hours after the solutions have become clear. Under these conditions the phosphotungstic acid does not interfere at all with the accuracy of the determination.

*Determination of Amino Nitrogen in the Filtrate from the Bases*  
Ten cubic centimeter portions of the filtrate are used for the amino determinations, which are run in the usual manner for six to ten minutes. The volume of nitrogen gas given off by a given amount of amino nitrogen is 2.5 times the volume of  $\frac{N}{16}$  acid neutralized if the same amount is determined by the Kjeldahl method. Therefore the portions (25 and 10 cc) taken for total and amino nitrogen determinations give results of similar accuracy. As 25-35 cc of gas or acid are usually measured, with an error not, as a rule, exceeding 0.2 cc, the percentage error of these determinations is small.

*Purity of Reagents* Since some of the calculations are based on differences between determinations, it is imperative that the latter shall be accurate. Every reagent used for either Kjeldahl or amino determination must be tested by blank analyses, and a correction applied in case any nitrogen is detected. A slight correction is usually necessary for both the commercial alkali used in distilling off the ammonia in the Kjeldahl determinations and for the sodium nitrite used in the amino determinations. We have also found potassium sulfate imported from one of the best known firms, 10 grams of which gave off ammonia by Kjeldahl sufficient to neutralize 2 cc of  $\frac{N}{16}$  acid. The accuracy of the standard solutions and apparatus used for both Kjeldahl and amino determination should likewise be carefully checked by determinations performed on pure substances. It is, of course, essential that pipettes, measuring flasks, and burettes should be calibrated.

The phosphotungstic acid used is purified with ether and water, by Winterstein's method.

*Corrections for Solubilities of the Bases* The solubility corrections are made from the following table, which can be applied directly when the bases are precipitated in the prescribed manner from a solution of 200 cc volume. It is true that the concentration of phosphotungstic acid left in solution, when the bases are precipitated, varies somewhat with the amount of the latter, so that the conditions of precipitation are not absolutely constant. It does not appear, however, that the variation is sufficient to cause significant change in the solubilities of the bases. When the latter are precipitated from solutions of greater or less volume than 200 cc, the solubility correction will, of course, vary in direct

proportion to the volume of the solution. The amount of nitrogen dissolved from the precipitate by the washing, performed in the manner described previously, is not, to judge from the control experiments, appreciable.

TABLE II

*Solubilities of the Bases in 200 cc of the Solution in which they are Precipitated*

	TOTAL N (ADD TO THE INDIVIDUAL BASES)	AMINO N	NON-AMINO N
	gram	gram	gram
Arginine N	0 0032	0 0008	0 0024
Histidine N	0 0038	0 0013	0 0025
Lysine N	0 0005	0 0005	0 0000
Cystine N	0 0026	0 0026	0 0000
Sum (subtract from figures for filtrate)		0 0052	0 0049

*Limits of Accuracy of the Determinations* The maximum and average differences found between duplicates in analyses of gliadin, edestin, hair, gelatin, fibrin, and hemocyanin are shown in the following table, expressed in percentages of the total nitrogen of the proteins.

TABLE III

	MAXIMUM DIFFERENCE BETWEEN DUPLICATES	AVERAGE DIFFERENCE
Ammonia N	0 37	0 12
Melanin N	0 39	0 20
Cystine N	0 11	0 05
Arginine N	1 27	0 73
Histidine N	2 14 (0 93)	0 79
Lysine N	1 23	0 61
Amino N in filtrate from bases	1 60 (0 60)	0 63
Non-amino N in filtrate	1 20	0 68

The maximum difference for the histidine (from edestin analysis) and that for amino nitrogen of the filtrate (from hair analysis) are more than twice as great as any other deviations found for these figures in the series. The next largest are given in parentheses, and represent as large differences between duplicates as are normally to be expected.

## TESTS OF THE METHODS ON PURE AMINO-ACIDS

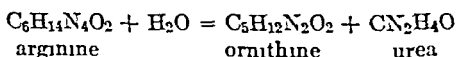
Experiments showing the manner in which all the natural amino-acids react when analyzed for *amino nitrogen* have been reported in the paper on the amino determination method, and therefore require no discussion here

The principle of *ammonia* determination by vacuum distillation is so well known that it likewise requires no especial discussion <sup>1</sup>

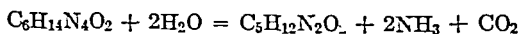
The apparatus shown in Fig 1 embodies no new principles, but is merely a simpler form which enables one to perform the determination without special apparatus. The 200 cc guard flask, to prevent escape of the ammonia which is not condensed in the receiving flask, is the only addition required to the vacuum distillation apparatus used for merely concentrating water solutions. In control analyses with standard ammonium sulphate solutions the method gave absolutely accurate results, and the determinations of amid nitrogen in the protein analyses always checked closely. Control determinations with 0.2 gram portions of cystine and of arginine carbonate showed that neither of these amino-acids gives off any ammonia under the conditions of the determination.

**ARGININE** The two steps in the determination of arginine are (1) the precipitation with phosphotungstic acid, (2) the decomposition with alkali.

Experiments were first undertaken to establish the conditions for the quantitative decomposition with alkali. Schulze and Winterstein<sup>2</sup> have shown that arginine when boiled in alkaline solution decomposes into ornithine and urea.



It is well known that urea, when boiled with either mineral acids or alkalis, is hydrolyzed into ammonia and carbon dioxide. Combining this reaction with the one written above, we have the following equation, which should represent the end products of the action of alkalis upon arginine if no side reactions occur.



<sup>1</sup> Cf Abderhalden *Biochemische Arbeitsmethoden*

<sup>2</sup> *Zeitschr f physiol Chem*, xxxiv, p 153, 1910

That is, *one-half of the arginine nitrogen is transformed into ammonia*. The possibility that this reaction might serve for determination of arginine is apparent from the work of Osborne, Leavenworth, and Brautlecht<sup>1</sup>. These authors found that when arginine was boiled down twelve to twenty times with  $\frac{N}{10}$  NaOH in a Kjeldahl flask it evolved ammonia at a gradually decreasing rate, the total amount representing the greater part of the ammonia indicated by the above equation. Also, when proteins were similarly treated they evolved amounts of ammonia approximately equivalent to the amid nitrogen of the proteins plus one-half the arginine nitrogen.

We found that by using stronger solutions of alkali the reaction could be made sharply quantitative. 0.2127 gram of the acid silver nitrate salt,  $C_6H_{14}N_4O_2 \cdot AgNO_3 \cdot HNO_3$ , was placed in a Kjeldahl flask with 50 cc of 50 per cent NaOH and 300 cc of water. The solution was boiled down to about 100 cc, then diluted and boiled down again. This process, performed five times, resulted in the evolution of the following amounts of  $\frac{N}{10}$  ammonia, 6.95, 2.00, 0.75, 0.48, and 0.20 cc, making in all 10.38 cc, while the theoretical amount is 10.45 cc. Repetition of the experiment gave similar results.

The method was, however, prolonged and inconvenient because of the number of times which the distillation had to be repeated, and because the solutions required constant watching in order to prevent too great concentration of the alkaline mixture and resultant destruction of the ornithine. The apparatus shown in Fig 2 was accordingly tried, with entirely satisfactory results.

**Experiment 1** Of the acid silver nitrate salt of arginine 0.2145 gram was dissolved in 25 cc of water in the Kjeldahl flask of the apparatus. Twenty-five cc of 50 per cent sodium hydrate solution was added, and the solution was kept boiling gently for five hours. The ammonia which collected in the Folin bulbs during this time neutralized 10.00 cc of  $\frac{N}{10}$  acid. One hundred cc of water were added to the solution, and the remainder of the ammonia distilled off as described in the directions for arginine determination. 0.35 cc of  $\frac{N}{10}$  acid was neutralized, making 10.35 cc in all, while the theoretical amount is 10.53 cc.

**Experiment 2** Of the same arginine salt 0.2031 gram was treated in the same manner, except that the boiling was continued for six hours. The ammonia collected in the Folin bulbs neutralized 9.75 cc of  $\frac{N}{10}$  acid, and the

<sup>1</sup> *Amer Journ of Physiol*, **XXIII**, p 180

subsequent distillation yielded an amount neutralizing 0.25 cc more, making 10.00 cc in all, the theoretical amount being 9.98 cc

Experiment 3 To a solution of 0.1758 gram of arginine carbonate and 30 cc of water, 15 grams of solid potassium hydrate were added, and the solution was boiled in the usual manner for five hours. The ammonia collected in the Folin bulbs neutralized 14.45 cc of  $\frac{N}{10}$  acid, that by the subsequent distillation 0.48 cc more making in all 14.93 cc, the theoretical amount being 15.02 cc

In order to determine whether the other amino-acids precipitated by phosphotungstic acid were stable under the conditions of the arginine determination, portions of lysine, histidine, and cystine were treated in the same manner as the arginine. The results in detail will be given in the sections devoted to the experiments with these acids. The lysine and histidine were found to give off no ammonia, while the cystine gave off regularly 17-18 per cent of its nitrogen in this form.

Gulewitsch<sup>1</sup> has found that the solubility of arginine phosphotungstate, under practically the same conditions used in the precipitation and washing of the bases here, is such that 2.2 mg of arginine nitrogen are dissolved in 100 cc of the phosphotungstate solution. In the following experiment we obtained the slightly smaller figure of 1.6 mg

0.215 gram of the acid silver nitrate salt of arginine was dissolved in water and precipitated at 200 cc dilution, in the presence of 5 per cent of sulphuric acid, with 12 grams of phosphotungstic acid. The solution was allowed to stand twenty-four hours at 20°, then filtered on a folded filter and allowed to drain. 175 cc of the filtrate were submitted to Kjeldahl determination, and neutralized 2.00 cc of  $\frac{N}{10}$  acid, indicating 2.8 mg of nitrogen in the 175 cc of filtrate, or 1.6 mg in 100 cc. As a check on the determination, the precipitate and the remainder of the filtrate were placed in a Kjeldahl flask with 300 cc of water and 50 cc of 50 per cent sodium hydrate solution. The mixture was boiled down repeatedly, as described in the first experiment on the arginine determination, and gave off during the successive distillations 6.86, 1.45, 0.73, and 0.30 cc of  $\frac{N}{10}$  ammonia, making in all 9.44 cc. This indicates 0.0264 gram of nitrogen, while the amount present, as calculated by difference from the total nitrogen content of the arginine and the nitrogen found in the filtrate, should be 0.0268 gram.

**HISTIDINE** 0.2 gram of pure histidine dichloride was precipitated at 20° in the same manner as the arginine. The histidine did not begin to precipi-

<sup>1</sup> *Zeitschr f physiol Chem*, xxvii, p 196

tate for some time after the phosphotungstic acid was added, but finally appeared in the form of shining crystals, which grew slowly on the bottom of the flask. After standing over night, the compact precipitate was filtered off. 100 cc of the filtrate were freed from phosphotungstic acid with barium hydrate, and the filtrate from the barium phosphotungstate was concentrated and submitted to Kjeldahl determination. The amount of  $\frac{N}{16}$  acid neutralized was 2.68 cc, indicating 3.68 mg of nitrogen in the 100 cc of filtrate. The remainder of the filtrate was allowed to stand another twenty-four hours, however, and a little more histidine phosphotungstate crystallized from it. This was filtered, without washing, and the filtrate, amounting to 95 cc, was treated in the same manner as the former portion. The amount of  $\frac{N}{16}$  acid neutralized was 1.30 cc, indicating 1.9 mg of nitrogen in 100 cc of the filtrate.

The above results show that at least forty-eight hours must be allowed for the complete precipitation of the histidine and that the solubility of the histidine under the conditions of the precipitation is about 1.9 mg of histidine nitrogen per 100 cc of solution.

The histidine precipitates were combined and boiled with alkali in the same manner as the arginine, but gave off no ammonia. Negative results were also obtained when 0.1 gram of histidine dichloride was boiled with alkali under a reflux as in the regular arginine determination.

**LYSINE** 0.2 gram of lysine picrate was precipitated at 200 cc volume in the same manner as the arginine and histidine. The lysine phosphotungstate, even in so dilute a solution, forms immediately, and the solubility determination shows that the lysine phosphotungstate is the most insoluble formed by any of the bases. 190 cc of the filtrate were freed from phosphotungstic acid with barium hydrate, and from excess of barium with an equivalent of sulfuric acid. The solution was then concentrated to a volume of a few cc, and used for determination of amino nitrogen. 0.9 cc of nitrogen gas at 751 mm,  $23^{\circ}$ , was given off. This indicates the presence of only 0.24 mg of lysine nitrogen in 100 cc of the filtrate.

The experiment was repeated in the same manner, except that the precipitation was performed in the presence of an equivalent concentration (3.5 per cent) of hydrochloric acid instead of 5 per cent sulphuric. 193 cc of the filtrate was freed from phosphotungstic acid by extraction with ether, according to the method of Winterstein,<sup>1</sup> and the solution was concentrated to dryness to drive off the hydrochloric acid. The residue was redissolved in a few cc of dilute sodium hydrate solution, and made up to 10 cc volume. 9.67 cc of this solution gave, on amino determination,

<sup>1</sup> *Zeitschr f physiol Chem*, xxiv p 153, 1901

0.84 cc of nitrogen gas at 768 mm and  $20^{\circ}$ . This indicates, as did the former determination, 0.24 mg of lysine nitrogen dissolved in 100 cc of the filtrate.

In order to test the stability of lysine under the conditions of the arginine determination, 0.2 gram of lysine picrate was dissolved in dilute sulfuric acid and freed from picric acid by extraction with ether. The solution was brought to 20 cc volume, and placed in the flask of the apparatus for arginine determination (Fig. 2). An equal volume of 50 per cent sodium hydrate was added, and the solution was boiled for 8 hours. No ammonia whatever was evolved.

**CYSTINE Experiment 1** 0.1 gram of cystine was dissolved in water containing 10 cc of concentrated hydrochloric acid, a solution containing 5 grams of phosphotungstic acid was added, and the entire solution was diluted to 100 cc. In a few minutes the cystine phosphotungstate began to separate in crystals similar to those formed by histidine. After twenty-four hours the precipitate was filtered on a small hardened filter paper and washed with suction several times, exactly in the manner described for treating the precipitate of the bases in the protein analysis. The precipitate was removed from the filter, dissolved in the requisite amount of dilute sodium hydrate, as described for the protein analysis, and the solution was freed from phosphotungstic acid with barium chloride. It was then brought to 50 cc volume, and determinations of the sulfur, amino nitrogen, and total nitrogen were made upon aliquot portions.

**Sulfur** Ten cc duplicate portions. The weights of  $\text{BaSO}_4$  were 0.0333 and 0.0332 gram, the average of the two indicating 10.0 milligrams of cystine nitrogen in the entire 50 cc of solution.

**Nitrogen** Twenty cc of the solution were used for Kjeldahl determination. The ammonia from the latter neutralized 2.95 cc of  $\frac{N}{16}$  acid, indicating 10.3 mgs of nitrogen in the total 50 cc.

**Amino Nitrogen** From 9.60 cc of the solution 3.80 cc of nitrogen gas at  $22^{\circ}$ , 760 mm were obtained. Multiplying this by the factor 0.92 to correct for the abnormal behavior of cystine with nitrous acid,<sup>1</sup> gives 3.50 cc of nitrogen from the cystine, indicating 10.1 cc of cystine nitrogen in the entire 50 cc.

**Nitrogen in the Filtrate** The filtrate and washings were digested together for Kjeldahl determination, air being drawn through the solutions, as suggested by Denis,<sup>2</sup> to prevent the bumping usually caused by the presence of a large amount of phosphotungstic acid during the digestion. The nitrogen present neutralized 0.95 cc of  $\frac{N}{16}$  acid, indicating 1.3 mg of nitrogen in the filtrate and washings from the cystine phosphotungstate precipitate. Added to the amount of cystine nitrogen in the precipitate, as determined

<sup>1</sup> This Journal, ix, p 199, 1911

<sup>2</sup> This Journal, viii, p 365



respectively by the three above methods, this gives 11.3, 11.6, or 11.4 mgs of cystine nitrogen regained out of the 11.66 mgs present in the 0.1 gram of cystine used. It also indicates that the amount of cystine nitrogen dissolved in 100 cc of solution under the conditions of the precipitation is not more than 1.3 mgs. A repetition of the solubility determination under the same conditions, except that only the filtrate, not the washings, was used for the Kjeldahl, gave the same result, 1.3 mg, indicating that the washing did not dissolve appreciable amounts of the precipitate. Determinations under the same conditions, except that they were made in the presence of 5 per cent sulfuric instead of 3.5 per cent hydrochloric acid gave the slightly higher solubility figure of 1.85 — 1.88 mg per 100 cc.

Although cystine is precipitated as completely as either arginine or histidine the amounts obtained from hydrolyzed proteins fell far short of what would be expected from the sulfur contents. It appeared possible that the cystine is partially destroyed during the hydrolysis. The results in the following table show that this is the case,—the cystine is gradually altered during acid hydrolysis into a substance or substances which are not precipitated by phosphotungstic acid.<sup>1</sup>

TABLE IV

HOURS BOILED WITH HCl	N IN 100 CC FILTRATE	N IN PRECIPITATE	METHOD OF DETERMINATION OF N IN PRECIPITATE	N IN FILTRATE CORRECTED FOR SOLUBILITY	PER CENT CYSTINE DESTROYED
	mg	mg		mg	per cent
0	1.3	10.2	S, N, NH <sub>2</sub>	0.0	0
8	4.6	7.0	Difference	3.3	29
16	6.0	5.6	Difference	4.7	41
24	7.1	4.53	N	5.8	50
		4.55	S		
		4.65	NH <sub>2</sub>		

*Experiment 2* One-tenth gram portions of cystine were boiled under a reflux condenser with 20 cc portions of 20 per cent hydrochloric acid for the lengths of time indicated in the table. The solutions were then transferred to 100 cc flasks, and 5 grams of phosphotungstic acid in solution added to each. The solutions were diluted with water to 100 cc each, and the cystine allowed to precipitate, as before, under practically the same conditions under which the bases precipitate in the protein analysis. The amount of phosphotungstic acid, 5 grams per 100 cc, was less than that used

<sup>1</sup> Morner found that boiling cystine 109 hours with 10 per cent hydrochloric acid reduced its specific rotation from  $-223^{\circ}$  to  $-134^{\circ}$ , and changed part of it into a form apparently more soluble than the natural cystine (*Zeitschr physiol Chem*, 1911, p 207)

in the protein analysis (7.5 grams) in order to allow for the fact that in the latter a portion of the phosphotungstic acid is withdrawn from solution by the other bases which are precipitated

It remains to test the behavior of cystine when boiled with alkali under the conditions of the arginine determination. The following tests were carried out under the exact conditions of the arginine determination, samples of cystine being boiled 6 hours with either 25 per cent sodium hydrate or 33 per cent potassium hydrate, the solutions being then diluted, and the residual ammonia boiled off on a Kjeldahl still

*Experiment 3* (a) One-tenth gram of cystine was boiled with 50 cc of 25 per cent "reagent" sodium hydrate, as described. The ammonia evolved neutralized 1.50 cc of  $\frac{N}{10}$  acid, equivalent to 2.10 mg of nitrogen, or 18.0 per cent of that present in the cystine

(b) The same experiment repeated yielded ammonia neutralizing 1.55 cc of  $\frac{N}{10}$  acid, equivalent to 18.6 per cent of the cystine nitrogen

(c) The same conditions were repeated, except that 0.2 gram of cystine was used. The ammonia neutralized 2.75 cc of  $\frac{N}{10}$  acid, equivalent to 16.5 per cent of the cystine nitrogen

(d) One-tenth of a gram of cystine was boiled six hours with a solution of 15 grams of potassium hydrate in 30 cc of water. The ammonia evolved neutralized 1.50 cc of  $\frac{N}{10}$  acid, equivalent to 18 per cent of the nitrogen present in the cystine

(e) One-tenth gram portions of cystine were boiled in the same manner with 50 cc portions of 25 per cent sodium hydrate, but in a *copper* flask. The amounts of  $\frac{N}{10}$  acid neutralized in three successive experiments were 5.03, 5.80, and 5.55 cc, equivalent to 60.3, 69.6, and 66.6 per cent, respectively, of the cystine nitrogen. Because of the effect of the copper in catalyzing the reaction that results in the evolution of ammonia from cystine copper flasks can not be used in the arginine determination

The cystine used in the above experiments was obtained from a bladder stone and purified by repeated solution in water, to which a minimum amount of sodium hydrate was added, followed by precipitation with acetic acid. The purity was controlled by determinations of the nitrogen, carbon, hydrogen, and sulfur

**TRYPTOPHANE** Tryptophane is known to be precipitated partially with phosphotungstic acid, even from fairly dilute solutions. When it is boiled with mineral acids, however, it is, to a large extent at least, destroyed, the nature and fate of the products being unknown. In order to ascertain the behavior of tryptophane under the conditions of the protein hydrolysis described in this paper, the following experiment was performed

Nine-tenths of a gram of tryptophane was boiled for twelve hours with 100 cc of 20 per cent hydrochloric acid. The solution was concentrated as dry as possible *in vacuo*, then redissolved in water. The solution was clear, free from insoluble matter, and but very slightly colored. It is evident that the *melanin* formed when proteins are hydrolyzed is not a decomposition product of tryptophane.

The *ammonia* determination also was negative.

After the latter, the solution was brought to 100 cc and determinations of the total and amino nitrogen made on portions of 10 cc each.

*Amino nitrogen* 11.00 cc of nitrogen gas at  $21^{\circ}$ , 756 mm, indicating 0.0620 gram of amino nitrogen in the total 100 cc of solution.

*Total nitrogen* In the Kjeldahl determination 8.90 cc of  $\frac{N}{10}$  acid were neutralized, indicating 0.1246 gram of nitrogen in the total solution.

The remaining 80 cc of solution were treated with phosphotungstic acid, in the regular manner for precipitating the bases, the solution being at 200 cc volume. A precipitate formed, even while the phosphotungstic acid was being added. When it was filtered, after 48 hours, the filtrate was not diluted with the washings, but portions of 40 and 25 cc respectively were used for Kjeldahl and amino nitrogen determinations. Before withdrawing the portions for determination of amino nitrogen, the solution was freed from air by shaking it in an evacuated flask, as the air in 25 cc of solution would necessitate a correction of 0.4 cc to the nitrogen gas. The results were:

*Amino nitrogen* 5.25 cc of nitrogen gas at  $22.5^{\circ}$ , 758 mm, indicating 0.0300 gram of amino nitrogen in the total filtrate (from 0.72 gram of tryptophane).

*Total nitrogen* 7.00 — 6.95, average, 6.98 cc of  $\frac{N}{10}$  acid, indicating 0.0611 gram of nitrogen in the total filtrate.

Both filtrate and precipitate, when portions of them were freed from phosphotungstic acid with barium hydrate and tested for tryptophane with glyoxylic acid, gave positive tests.

The main portion of the precipitate was boiled with 25 per cent sodium hydrate solution in the apparatus for arginine determination, it gave off no ammonia.

The above facts lead to the following conclusion:

- 1 The tryptophane is responsible for none of the nitrogen estimated as ammonia, arginine, or melanin.

- 2 Boiling with 20 per cent hydrochloric acid does not alter the ratio 2 : 1 of total nitrogen to amino nitrogen in tryptophane.

- 3 Tryptophane, under the conditions of the protein analysis, can be precipitated with the "base" fraction. The amount of tryptophane taken, however, was equivalent to 30 per cent of the total amount of protein used in an analysis. Even of this large amount, only 38.7 per cent was precipitated by the phospho-

tungstic acid, that is, an amount equal to about 20 per cent of a protein, as ordinarily analyzed, remained unprecipitated. Of tryptophane which is precipitated three-fourths would, in the protein analysis, be calculated as histidine, the other fourth as lysine. In order to determine whether tryptophane were actually present in the phosphotungstic precipitated from any of the proteins analyzed (cf. latter portion of this paper), a portion of the solution of the precipitated amino-acids was tested in each case for tryptophane with glyoxylic acid. Although the control experiment cited above indicates that the tryptophane or products thereof precipitated gives the glyoxylic test, none of the precipitates yielded by the proteins gave any trace of it. It appears improbable that tryptophane affects the composition of the phosphotungstic precipitate under the usual condition of the analysis, but it is advisable in the latter, as a precaution, to test a few drops of the solution of the bases for tryptophane.

**ANALYSIS OF AN ARTIFICIAL MIXTURE OF AMINO-ACIDS** In order to obtain a mixture of complexity similar to that obtained by hydrolyzing proteins, the following were dissolved together: of aspartic acid, glutamic acid, proline, oxyproline, phenylalanine, tyrosine, alanine, glycocoll, valine, leucine, cystine, histidine dichloride, and ammonium sulfate, each 0.200 gram. Of arginine carbonate, 0.2137 gram was taken. For the lysine, 0.75 gram of lysine picrate was dissolved in dilute hydrochloric acid and freed from picric acid by extracting with ether. The solution was then concentrated and brought to 50 cc volume. For a Kjeldahl determination 9.50 cc were used, and 6.73 cc of  $\frac{N}{10}$  acid were neutralized. Forty cubic centimeters of the solution, containing 0.0395 gram of lysine nitrogen were added to the mixture of amino-acids. The nitrogen contents of the substances present add up to 0.4266 gram of nitrogen. The solution was brought to 100 cc and aliquot parts of 5 cc each were used for Kjeldahl determinations. The amounts of  $\frac{N}{10}$  acid neutralized were 15.24 and 15.39 cc, indicating 0.4267 and 0.431 gram of nitrogen respectively in the entire solution.

The solution was analyzed in exactly the manner described for protein analysis. Ninety cubic centimeters of the original 100 cc of solution were used, the nitrogen results being calculated to apply to the total 100 cc.

*Ammonia* 27.8 cc of  $\frac{N}{10}$  acid were neutralized, indicating 0.0432 gram of ammonia nitrogen in the original total 100 cc of solution

*Cystine* The  $\text{BaSO}_4$  weighed 0.0618 gram, indicating 0.0206 gram of cystine nitrogen in the total solution

*Arginine* 8.89 cc of  $\frac{N}{10}$  acid were neutralized. A correction of 1.09 cc must be applied for the cystine, leaving 7.80 cc for the arginine, indicating 0.0485 gram of arginine nitrogen

*Total Nitrogen of the Phosphotungstate Precipitate or "Bases"* The solution which had been used for the arginine determination neutralized, by Kjeldahl method, 35.78 cc of  $\frac{N}{10}$  acid. This, added to the 8.89 cc of the arginine determination, gives 44.67 cc, indicating 0.1390 gram of nitrogen in the amino-acids precipitated by phosphotungstic

*Amino Nitrogen of the Phosphotungstate Precipitate, or "Bases"* The amino determination yielded 26.3 cc of nitrogen gas at  $19^\circ$ , 768 mm, indicating 0.0845 gram of amino nitrogen. The cystine introduces a correction of 0.0014 gram, leaving 0.0831 gram

*Amino Nitrogen of the Filtrate from the Bases* The determinations gave

(I) 20.8 cc of nitrogen gas at  $21^\circ$ , 766 mm, indicating 0.1975 gram of amino nitrogen in the filtrate

(II) 20.7 cc of nitrogen gas at  $22^\circ$ , 766 mm, indicating 0.1960 gram of amino nitrogen in the filtrate. The average is 0.1968 gram

*Total Nitrogen in the Filtrate* The duplicate Kjeldahl determinations neutralized 26.27 and 26.20 cc of  $\frac{N}{10}$  acid, the average indicating 0.2450 gram of nitrogen in the filtrate

The results are summarized in the following table, the histidine and lysine being calculated as described previously

TABLE V

	PRESENT	FOUND (UNCORRECTED)	FOUND (CORRECTED FOR THE SOLUBILITY OF THE BASES)
Total N	0.4266	0.4289 (Kjeldahl)	
Ammonia N	0.0424	0.0432	0.0432
Arginine N	0.0511	0.0485	0.0517
Cystine N	0.0233	0.0206	0.0232
Histidine N	0.0369	0.0294	0.0332
Lysine N	0.0396	0.0405	0.0410
Amino Nitrogen of the filtrate from the bases	0.1867	0.1968	0.1916
Non-amino N of the filtrate (proline and oxyproline)	0.0457	0.0482	0.0433
Total N regained (sum)	0.4266	0.4272	0.4272

The agreement between the nitrogen figures found and those calculated from the amounts of amino-acids present is fairly satisfactory, especially when the solubility corrections for the bases are applied. The mixture contained all of the amino-acids known to occur commonly in proteins except tryptophane. The results indicate about the same degree of accuracy as the agreement between the duplicates in the protein analyses reported in the latter portion of this paper. The test of the method would be made more complete by analysis of a mixture like the above, but containing tryptophane and boiled 24 hours with 20 per cent hydrochloric acid. Unfortunately the completion of this experiment was prevented by the expiration of the period available for the work, which can not be taken up again for a number of months. The consistency of the results obtained, however, both in experiments with pure amino-acids and in analyses of proteins, indicates, with a fair degree of conclusiveness, the reliability of the method.

#### ANALYSES OF PROTEINS

##### *Gladin*

Ten grams of gladin from wheat were dissolved in about 60 cc of 20 per cent hydrochloric acid and boiled under a reflux in a tared flask. After twenty hours a sample of 1 cc was withdrawn, diluted to 10 cc, and used for determination of amino nitrogen. 9.65 cc of this solution yielded 20.0 cc of nitrogen gas at 17°, 763 mm, indicating 23.81 mg of nitrogen gas from the entire 10 cc. The weight of the hydrolyzing solution after the sample was withdrawn was 81.7 grams. After eight hours more boiling the solution had concentrated to 79.5 grams. If the amino nitrogen had remained constant, 1 cc should now yield 24.45 mg of nitrogen gas. The amount obtained was 26.45 mg, showing that additional hydrolysis had occurred since the preceding determination. The hydrolysis was continued for ten hours more, during which the weight of the solution decreased from 78.4 to 77.6 grams. The amount of nitrogen gas to be expected from 1 cc of this solution, if no further hydrolysis had occurred, was 26.72 mg. The amount obtained was 26.92 mg, showing that the hydrolysis had been complete at the end of 28 hours.

The solution was diluted to 250 cc and 10 cc samples taken for Kjeldahl determinations. The amounts of  $\frac{N}{10}$  acid neutralized were 43.00 and 43.05 cc, the average indicating a nitrogen content of 0.452 gram in the 75 cc portions of the solution used for the subsequent analyses.

The analyses were performed on duplicate solutions, the details corresponding in all respects to the description of the method in the early part of the paper. The results follow.

*Ammonia* The amounts of  $\frac{N}{10}$  acid neutralized were 82.78 and 81.9 cc, indicating 0.116 and 0.1147 gram of amid nitrogen.

*Melanin* The amounts of  $\frac{N}{10}$  acid neutralized were 2.80 and 2.80 cc, indicating 0.0039 gram of nitrogen.

*Cystine* The  $\text{BaSO}_4$  weighed 0.0086 and 0.0097 gram, indicating 0.0026 and 0.0029 gram of cystine nitrogen. The amount of cystine here, as in most of the proteins analyzed, is too small to affect significantly the figures for the arginine or amino nitrogen.

*Arginine* The amounts of  $\frac{N}{10}$  acid neutralized were 4.11 and 3.97 cc, indicating 0.0230 and 0.0223 gram of arginine nitrogen.

*Total Nitrogen of the Bases* The amounts of  $\frac{N}{10}$  acid neutralized by the Kjeldahl determinations were 13.39 and 12.75 cc. Added to the amounts neutralized by the ammonia evolved in the arginine determinations these give 17.50 and 16.72 cc, indicating 0.0490 and 0.0468 gram of nitrogen.

*Amino Nitrogen of the Bases* The amounts of nitrogen gas evolved were 6.50 cc at  $23^\circ$ , 760, and 6.00 cc at  $21^\circ$ , 760 mm. These indicate 0.0183 and 0.0170 gram of amino nitrogen.

*Amino Nitrogen of the Filtrate from the Bases* The amounts of nitrogen gas were (I) 28.8 – 28.7 cc at  $23^\circ$ , 748 mm, indicating 0.2399 gram of amino nitrogen in the filtrate, (II) 29.0 – 29.1 cc of gas at  $24^\circ$ , 748 mm, indicating 0.2414 gram of nitrogen.

*Total Nitrogen of the Filtrate* The amounts of  $\frac{N}{10}$  acid neutralized were (I) 33.43 – 33.63 cc, indicating 0.2820 gram of nitrogen, (II) 33.93 – 34.13 cc, indicating 0.2860 gram of nitrogen.

The results calculated from these data are collected in the table on the following page.

The figures for the amino and non-amino nitrogen of the filtrate under "results by other methods" are calculated from the isolated amounts of the amino-acids which are listed in Table I as belonging to these fractions. The methods of isolation are known to be accompanied by unavoidable losses. Their extent is indicated by comparison with the results of the group determination method, which furnishes a criterion for the completeness of the isolations,

TABLE VI

*Results of Gliadin Analysis in Percentages of Total Nitrogen*

	I	II	AVERAGE	AVERAGE CORRECTED FOR SOLUBILITY OF BASES	RESULTS BY OTHER METHODS
Ammonia N	25 86	25 37	25 52		25 2†, 19 51‡
Melanin N	0 86	0 86	0 86		
Cystine N	0 72	0 63	0 68	1 25	
Arginine N	5 10	4 93	5 01	5 71	6 44,* 6 00†, 5 1‡
Histidine N	4 42	4 28	4 36	5 20	2 7,* 0 9,† 1 9†
Lysine N	0 82	0 43	0 64	0 75	0 0,* 0 0,† 0 0†
Amino N in filtrate	52 85	53 40	53 13	51 98	28 5,* 29 9†
Non-amino N in filtrate	9 29	9 86	9 58	8 50	2 6,* 5 1†
Total re- gained	99 72	99 76	99 78		

\*Abderhalden and Samuely *Zeitschr f physiol Chem* xliv p 282†Osborne and Clapp *Amer Journ of physiol* xvii p 231‡Kossel and Kutscher *Zeitschr f physiol Chem* xxxi p 201

That a part of the large deficit in the latter may be due to unknown  $\alpha$ -amino-acids is, of course, not impossible

### *Edestin*

Two separate portions of edestin were hydrolyzed, the course of the hydrolysis being followed as in the case of gliadin, by amino determinations until the latter showed constant results, which occurred after about 15 hours. The solutions used for the following analyses contained 0.3848 and 0.3305 gram of nitrogen respectively.

*Ammonia* The amounts of  $\frac{N}{16}$  acid neutralized were 26.93 and 24.03 cc., indicating 0.0377 and 0.0336 gram of nitrogen.

*Melanin* The amounts of  $\frac{N}{16}$  acid neutralized were 4.24 and 5.23 cc., indicating 0.0064 and 0.0073 gram of nitrogen.

*Cystine* The  $\text{BaSO}_4$  weighed 0.0090 gram in each case, indicating 0.0027 gram of cystine nitrogen.

*Arginine* The amounts of  $\frac{N}{16}$  acid neutralized were 18.53 and 15.02 cc., indicating 0.1033 and 0.0841 gram of arginine nitrogen.



*Total Nitrogen of the Bases* The amounts of  $\frac{N}{10}$  acid neutralized by the Kjeldahl determinations were 29.22 and 27.25 cc. Added to the amounts neutralized by the ammonia in the arginine determination, these give 47.75 and 42.27 cc. indicating 0.1337 and 0.1184 gram of nitrogen.

*Amino Nitrogen of the Bases* The amounts of nitrogen gas measured were (I) 16.4 cc. at  $19^{\circ}$ , 762 mm., indicating 0.0470 gram of nitrogen, (II) 15.27 cc. at  $22^{\circ}$ , 758 mm., indicating 0.0429 gram of nitrogen.

*Amino Nitrogen of the Filtrate* The amounts of nitrogen gas measured were (I) 22.0 cc. at  $18^{\circ}$ , 762 mm., indicating 0.1896 gram of nitrogen, and 22.3 cc., at  $20^{\circ}$ , 762 mm. indicating 0.1909 gram, (II) 18.80 cc. at  $19^{\circ}$ , 762 mm., indicating 0.1616 gram of nitrogen, and 18.90 cc. at  $22^{\circ}$ , 758 mm., indicating 0.1594 gram.

*Total Nitrogen of the Filtrate* The amounts of  $\frac{N}{10}$  acid neutralized were (I) 24.22 cc., indicating 0.2034 gram of nitrogen, (II) 20.1 and 20.3 cc., the average, 20.2, indicating 0.1698 gram of nitrogen.

TABLE VII

*Results of Edestin Analysis in Percentages of the Total Nitrogen*

	I		II		AVERAGE OF CORRECTED RESULTS	RESULTS BY OTHER METHODS
	UNCORRECTED	CORRECTED FOR SOLUBILITY OF BASES	UNCORRECTED	CORRECTED FOR SOLUBILITY OF BASES		
Ammonia N	9.80		10.17		9.99	
Melanin N	1.83		2.12		1.98	
Cystine N	0.71	1.38	0.82	1.60	1.49	1.6†
Arginine N	26.85	27.68	25.45	26.41	27.05	24.7-25.4*
Histidine N	3.63	4.63	5.63	6.77	5.75	3.5*
Lysine N	3.58	3.72	3.93	4.01	3.86	1.8*
Amino N of the Fil- trate	49.4	48.1	48.6	47.0	47.55	20.7†
Non-amino N in Fil- trate	3.5	2.1	2.8	1.3	1.7	0.7†
Sum	99.30		99.52		99.37	

\*Kossel and Patton *Zeitschr f physiol Chem* xxviii p 43

†Abderhalden *Zeitschr f physiol Chem*, xxxvii p 499

*Hair*

Ten grams of dog's hair were hydrolyzed, in the same manner as the gliadin, and the solution brought to 250 cc. Of this, 75 cc portions, containing, according to Kjeldahl determinations 0.4495 gram of nitrogen each, (10 cc of solution neutralized 35.64-35.72 cc of  $\frac{N}{10}$  acid) were used for the following duplicates

*Ammonia* The ammonia neutralized 32.07 and 32.05 cc of  $\frac{N}{10}$  acid, indicating 0.0449 and 0.0449 gram of amid nitrogen

*Melanin* The amounts of  $\frac{N}{10}$  acid neutralized were 23.98 and 23.58 cc, indicating 0.0336 and 0.0330 gram of nitrogen

*Cystine* The weights of  $\text{BaSO}_4$  were 0.0898 and 0.0910 gram, indicating 0.0269 and 0.0273 gram of cystine nitrogen

*Arginine* The amounts of  $\frac{N}{10}$  acid neutralized were 13.39 and 13.37 cc, or, subtracting the corrections for the amounts of ammonia evolved by the unusually large amount of cystine, 11.76 and 11.71 cc, indicating 0.0659 and 0.0656 gram of arginine nitrogen

*Total Nitrogen of the Bases* The amounts of  $\frac{N}{10}$  acid neutralized by the Kjeldahl determinations were 32.34 and 32.53 cc, which, added to the amounts previously neutralized by the ammonia from the arginine and cystine, give 45.73 and 45.89 cc, indicating 0.1281 and 0.1286 gram of nitrogen

*Amino Nitrogen of the Bases* The amounts of nitrogen gas evolved were (I) 26.20 cc at  $21^\circ$ , 776 mm, (II) 25.30 cc at  $20^\circ$ , 760 mm. These indicate 0.0740 and 0.0718 grams of amino nitrogen, or 0.0722 and 0.0700 grams when corrected for the abnormal behavior of cystine with nitrous acid

*Amino Nitrogen of the Filtrate* The amounts of nitrogen gas were (I) 25.7 - 25.4 cc at  $20^\circ$ , 770 mm, indicating 0.2207 gram of nitrogen, (II) 25.50 cc at  $19^\circ$ , 760 mm, and 25.10 cc at  $20^\circ$ , 760 mm, indicating 0.2157 and 0.2162 gram of nitrogen

*Total Nitrogen of the Filtrate* The amounts of  $\frac{N}{10}$  acid neutralized were 28.38 - 28.31 and 28.25 - 28.20 cc, indicating 0.2382 and 0.2373 gram of nitrogen

TABLE VIII

*Results of Dog's Hair Analysis in Percentages of the Total Nitrogen*

	I	II	AVERAGE	AVERAGE CORRECTED FOR SOLUBILITY OF BASES
Ammonia N	10 10	9 99	10 05	
Melanin N	7 35	7 48	7 42	
Cystine N	6 08	5 99	6 04	6 60
Arginine N	14 68	14 57	14 62	15 33
Histidine N	2 20	3 13	2 67	3 48
Lysine N	5 72	4 80	5 26	5 37
Amino N of filtrate	49 5	47 9	48 7	47 5
Non-amino N of filtrate	3 6	4 8	4 2	3 1
	28 7	28 5	28 6	30 8
	53 1	52 7	52 9	50 6
Total regained	99 23	98 46	98 96	

*Gelatin*

Ten grams of gelatin were hydrolyzed, as before described, and the solution brought to 250 cc. Ten cubic centimeter portions were used for Kjeldahl determinations. The amounts of  $\frac{N}{10}$  acid neutralized were 40.95 and 40.50 cc, indicating 0.428 gram of nitrogen in each of the 75 cc portions used for the following duplicate determinations.

*Ammonia* The ammonia neutralized 7.00 and 6.85 cc, of  $\frac{N}{10}$  acid, indicating 0.0098 and 0.0096 gram of ammonia nitrogen.

*Melanin* The amounts of  $\frac{N}{10}$  acid neutralized were 2.0 cc in each case, indicating 0.0028 gram of nitrogen.

*Cystine* The sulfur determinations were negative.

*Arginine* The amounts of  $\frac{N}{10}$  acid neutralized were 11.10 and 10.30 cc, indicating 0.0621 and 0.0577 gram of arginine nitrogen.

*Total Nitrogen of the Bases* The Kjeldahl determinations neutralized 25.55 and 25.60 cc of  $\frac{N}{10}$  acid. Added to the amounts neutralized in the arginine determinations, these give 36.65 and 35.90 cc, indicating 0.1027 and 0.1006 gram of nitrogen.

*Amino Nitrogen of the Bases* The amounts of nitrogen evolved were 16.1 cc at 17°, 758 mm, and 16.3 cc at 19°, 758 mm, indicating 0.0464 and 0.0467 gram of amino nitrogen.

*Amino Nitrogen of the Filtrate* The amounts of nitrogen evolved were (I) 29.25 – 29.45 cc at 24°, 750 mm, the average indicating 0.2428 gram of amino nitrogen, (II) 29.50 at 23°, 750 mm and 29.40 cc at 22°, 750 mm, both indicating 0.245 gram of amino nitrogen.

*Total Nitrogen of the Filtrate* The amounts of  $\frac{N}{10}$  acid neutralized were 37.20 – 37.00 and 37.28 – 37.28 cc, indicating respectively 0.3118 and 0.3130 gram of nitrogen

TABLE IX

*Results of Gelatin Analysis in Percentages of the Total Nitrogen*

	I	II	AVERAGE	AVERAGE CORRECTED FOR SOLUBILITY OF BASES	RESULTS BY OTHER METHODS
Ammonia N	2.30	2.20	2.25		2.2*
Melanin N	0.07	0.07	0.07		
Cystine N	0.00	0.00	0.00		
Arginine N	14.52	13.48	14.00	14.74	14.3–16.6*
Histidine N	3.39	3.79	3.59	4.48	
Lysine N	6.10	6.31	6.20	6.32	
Amino N of the filtrate	56.7	57.3	57.0	55.8	27†
Non-amino N of filtrate	16.1	15.9	16.0	14.9	9.5††
Total re- gained	99.18	99.05	99.11		

\* Kossel and Kutscher *Zeit. chr. f. physiol. Chem.* xxxi p. 204

† Levene and Beatty *Ibid.* xlix p. 261

‡ Fischer and Boehner *Ibid.* lxxv p. 118

The unusually high percentage of non-amino nitrogen in the filtrate indicates an exceptional amount of proline and oxyproline, which have, in fact, been isolated in previous hydrolyses by Levene and Beatty, and by Fischer and Boehner

### Fibrin

Ten grams of Merck's fibrin were hydrolyzed in the usual manner, and the solution was brought to 250 cc volume. Ten cubic centimeter portions taken for Kjeldahl determinations neutralized 42.00–42.15 cc of  $\frac{N}{10}$  acid, indicating 0.4415 gram of nitrogen in each of the 75 cc samples used for the following determinations

*Ammonia* The amounts of  $\frac{N}{10}$  acid neutralized were 26.0 and 26.4 cc, indicating 0.0364 and 0.0370 gram of ammonia nitrogen

*Melanin* The amounts of  $\frac{N}{10}$  acid neutralized were 10.8 and 9.08 cc, indicating 0.0151 and 0.0127 gram of nitrogen

*Cystine* The amounts of  $\text{BaSO}_4$  were 0.0059 and 0.0070 gram, indicating 0.0018 and 0.0021 gram of cystine nitrogen

*Arginine* The amounts of  $\frac{N}{10}$  acid neutralized were 10.72 and 9.83 cc, indicating 0.0600 and 0.0551 gram of arginine nitrogen

*Total Nitrogen of the Bases* The amounts of  $\frac{N}{10}$  acid neutralized were 34.3 and 36.0 cc. When added to the amounts neutralized in the arginine determinations, these give 45.0 and 45.8 cc, indicating total basic nitrogen of 0.1261 and 0.1284 gram

*Amino Nitrogen of the Bases* The amounts of nitrogen gas were 24.1 and 25.0 cc at  $21^\circ$ , 772 mm, indicating 0.0693 and 0.0719 gram of amino nitrogen

*Amino Nitrogen of the Filtrate from the Bases* The amounts of nitrogen were (I) 28.2 – 28.1 cc at  $17^\circ$ , 760 mm, indicating 0.244 gram of amino nitrogen, (II) 27.7 – 27.9 cc at  $13^\circ$ , 768 mm, indicating 0.2452 gram of amino nitrogen

*Total Nitrogen of the Filtrate* The amounts of  $\frac{N}{10}$  acid neutralized were 31.3 – 31.5 and 30.9 – 31.0 cc, indicating respectively 0.2638 and 0.2597 gram of nitrogen

TABLE X

*Results of Fibrin Analysis in Percentages of the Total Nitrogen*

	I	II	AVERAGE	AVERAGE CORRECTED FOR SOLUBILITY OF BASES
Ammonia N	8.24	8.38	8.32	
Melanin N	3.43	2.87	3.17	
Cystine N	0.41	0.45	0.43	0.99
Arginine N	13.60	12.48	13.14	13.86
Histidine N	3.78	4.14	3.96	4.83
Lysine N	10.78	12.01	11.40	11.51
Amino N in filtrate	55.3	55.5	55.4	54.2
Non-amino N in filtrate	4.4	3.3	3.85	2.7
Total N recovered	99.94	99.13	99.67	

### *Hemocyanin of Limulus*

The hemocyanin in the blood of *Limulus*, the horse-shoe crab, takes the place of hemoglobin in the blood of mammals. The specimen analyzed was kindly furnished by Dr. C. L. Alsberg, from material prepared at Woods Hole. Seven grams of the protein were hydrolyzed, and the solution made up to 250 cc. Por-

tions of 20 cc were taken for Kjeldahl determinations. The amounts of  $\frac{N}{16}$  acid neutralized were 64.1 and 64.0 cc, indicating 0.449 gram of nitrogen in each of the samples of 100 cc used in the following determinations:

*Ammonia* The amounts of  $\frac{N}{16}$  acid neutralized were 19.10 and 19.05 cc, indicating in both duplicates 0.0267 gram of ammonia nitrogen.

*Melanin* The amounts of  $\frac{N}{16}$  acid neutralized were 5.1 and 5.5 cc, indicating 0.0071 and 0.0077 gram of nitrogen.

*Cystine* The weights of barium sulphate were 0.0032 and 0.0035 gram, indicating in each case 0.0010 gram of cystine nitrogen in the precipitate of the bases.

*Arginine* The amounts of  $\frac{N}{16}$  acid neutralized were 11.80 and 12.30 cc, indicating 0.0661 and 0.0688 gram of arginine nitrogen.

*Total Nitrogen of the Bases* The amounts of  $\frac{N}{16}$  acid neutralized in the Kjeldahl determinations were 45.35 and 46.0 cc. These, added to the amounts neutralized in the arginine determinations, give 57.15 and 58.3 cc, indicating 0.1601 and 0.1632 gram of nitrogen in the precipitate of the bases.

*Amino Nitrogen of the Bases* The amounts of nitrogen gas obtained were 25.8 and 25.4 cc at 20° and 772 mm, indicating 0.0746 and 0.0734 gram of amino nitrogen.

*Amino Nitrogen of the Filtrate* The amounts of nitrogen gas obtained were 27.4 – 27.6 cc at 21°, 768 mm, indicating 0.2362 gram of amino nitrogen, and 28.15 – 27.99 cc at 21°, 749 mm, indicating 0.2348 gram of nitrogen.

*Total Nitrogen of the Filtrate* The amounts of  $\frac{N}{16}$  acid neutralized were 30.55 – 30.65 and 30.65 – 30.75 cc, indicating 0.2570 and 0.2580 gram of nitrogen in the filtrate from the bases.

TABLE XI

Results of Hemocyanin Analysis in Percentages of the Total Nitrogen

	I	II	AVERAGE	AVERAGE CORRECTED FOR SOLUBILITY OF BASES
Ammonia N	5.95	5.95	5.95	
Melanin N	1.59	1.71	1.65	
Cystine N	0.22	0.22	0.22	0.80
Arginine N	14.72	15.33	15.03	15.73
Histidine N	12.00	12.77	12.39	13.23
Lysine N	8.71	8.04	8.38	8.49
Amino N in filtrate	52.6	52.3	52.4	51.3
Non-amino N in filtrate	4.6	5.2	4.9	3.8
	57.2	57.5	57.3	55.1
Total N regained	100.39	101.52	100.92	

*Ox Hemoglobin*

The specimen of pure, crystallized hemoglobin for this analysis was kindly furnished me by Dr Butterfield of this Institute

Because of the relatively small amount of the material the details of the analysis had to be somewhat modified, in that the bases were precipitated from a solution of 100 cc instead of 200 cc volume, and the filtrate from the bases was brought to 100 cc instead of 150 before the aliquot portions for nitrogen determinations were taken Also, the total nitrogen, 0.1740 gram, on the basis of which the percentages are calculated, was obtained by addition of the ammonia, melanin, basic nitrogen, and nitrogen of the filtrate, instead of taking a separate portion of the solution for a Kjeldahl determination at the beginning of the analysis In other respects the details did not differ from those of the preceding analyses

The hemoglobin, which weighed a little more than a gram air-dried, was hydrolyzed for 28 hours

*Ammonia* The ammonia neutralized 6.50 cc of  $\frac{N}{10}$  acid, indicating 0.0091 gram of ammonia nitrogen

*Melanin* The amount of  $\frac{N}{10}$  acid neutralized was 4.53 cc, indicating 0.0063 gram of nitrogen

*Cystine* The weight of barium sulphate was less than 1 mg, which is considered a negative result

*Arginine* The amount of  $\frac{N}{10}$  acid neutralized was 2.12 cc, indicating 0.0118 gram of arginine nitrogen

*Total Nitrogen of the Bases* The amount of  $\frac{N}{10}$  acid neutralized in the Kjeldahl determination was 15.87 cc Added to the amount neutralized in the arginine determination, this gives 17.99 cc, indicating 0.0504 gram of nitrogen in the precipitate of the bases

*Amino Nitrogen of the Bases* The amount of nitrogen gas obtained was 10.05 cc at  $21^{\circ}$ , 752 mm, indicating 0.028 gram of nitrogen

*Amino Nitrogen of the Filtrate* The amounts of nitrogen gas obtained were 18.23 cc at  $21^{\circ}$ , 749 mm, and 18.09 cc at  $21^{\circ}$ , 749 mm These indicate 0.1017 and 0.0995 gram of amino nitrogen, or an average of 0.1006

*Total Nitrogen of the Filtrate* The duplicates each neutralized 19.35 cc of  $\frac{N}{10}$  acid, indicating 0.1083 gram of nitrogen

TABLE XII

*Results of Ox Hemoglobin Analysis in Percentages of the Total Nitrogen*

	UNCORRECTED	CORRECTED FOR SOLUBILITY OF BASES
Ammonia N	5.24	
Melanin N	3.6	
Arginine N	6.8	7.7
Cystine N	0.00	?
Histidine N	11.6	12.7
Lysine N	10.6	10.9
Amino N in filtrate	57.8	57.0
Non-amino N in filtrate	4.3	2.9
	29.0	31.3
	62.1	59.9
Total N*	(99.9)	

\*The total nitrogen in this case necessarily adds up to  $100 \pm 0.1$  per cent because the total nitrogen obtained by addition is used as the basis for calculating the other percentages. It does not therefore serve as a control for the accuracy of the hemoglobin analysis.

From hydrolysis of *horse* hemoglobin Abderhalden obtained 9.8 per cent of arginine nitrogen, 16.7 per cent of histidine nitrogen, 4.6 per cent of lysine nitrogen, 27.4 per cent of primary mono-amino-acid nitrogen, and 2.2 per cent of proline and oxyproline nitrogen<sup>1</sup>. The high histidine content is a striking characteristic of both ox and horse hemoglobin, as well as of *Limulus* hemocyanin.

In the following table the average results of the protein analyses, corrected for the solubilities of the bases, are collected

<sup>1</sup> *Zeitschr f physiol Chem*, xxvii, p 492, 1903



TABLE XIII

*Summary of Corrected Results in Percentages of the Total Nitrogen of the Proteins*

	GLIADIN	EDS- TIN	HAIR (DOG)	GELA- TIN	FIBRIN	HEMO- CYANIN	OF HEMOGLOBIN
Ammonia N	25 52	9 99	10 05	2 25	8 32	5 95	5 24
Melanine N	0 86	1 98	7 42	0 07	3 17	1 65	3 6
Cystine N	1 25	1 49	6 60	0 ?	0 99	0 80	0 ?
Arginine N	5 71	27 05	15 33	14 70	13 86	15 73	7 7
Histidine N	5 20	5 75	3 48	4 48	4 83	13 23	12 7
Lysine N	0 75	3 86	5 37	6 32	11 51	8 49	10 9
Amino N of the filtrate	51 98	47 55	47 5	56 3	54 3	51 3	57 0
Non-amino N of the filtrate (proline, oxy- proline, $\frac{1}{2}$ trypto- phane)	8 50	1 7	3 1	14 9	2 7	3 8	2 9
Sum	99 77	99 37	98 85	99 02	99 58	100 95	(100 0)*

\*Cf note to preceding table

The question marks in the decimal places for the figures on the cystine of gelatin and hemoglobin indicate that no cystine was found in the precipitate of the bases, but that a fraction of a per cent may have been present in the filtrate. The small amount of lysine obtained in the analysis of gliadin practically falls within the limit of experimental error, and is not to be regarded as contradictory to the negative results of previous authors<sup>1</sup>. The marked individual peculiarities of the different proteins, such as the high ammonia and low lysine of gliadin, the low ammonia and high non-amino nitrogen of the filtrate of gelatin, the high histidine of hemoglobin and hemocyanin, are apparent on inspection of the above table.

#### SUMMARY

Methods are developed for following the course of protein hydrolysis and detecting the time at which it is complete, and for determining the *ammonia* (amid nitrogen), *arginine*, *histidine*, *lysine*, *cystine*, *amino nitrogen not precipitated by phosphotungstic acid*

<sup>1</sup> *Loc cit*

(the group of ordinary primary amino-acids, leucine, alanine, etc.), and the *non-amino nitrogen not precipitated by phosphotungstic acid* (proline, oxyproline, one-half the tryptophane nitrogen) The scheme of analysis is outlined on pages 17-18

The analysis require but 2.5 to 3 grams of protein, and indicates the nature of 98 to 100 per cent of the nitrogenous products of hydrolysis The results of hydrolyses of wheat gliadin, edestin from hemp seed, dog's hair, gelatin, fibrin, hemocyanin (the protein from the blood of the king-crab corresponding to the hemoglobin of higher animals), and of ox hemoglobin are summarized in the last table The limits of accuracy of the determinations are indicated by the figures in Table III (page 32)

Comparison of the amounts of amino-acids of the different groups present in proteins, found by the quantitative group determination method, with the amounts isolated in previous hydrolyses, indicates that the losses of isolation have fallen chiefly on the primary mono- $\alpha$ -amino-acids, containing all their nitrogen in the  $-\text{CH}(\text{NH}_2)-\text{COOH}$  group So far as can be judged from the analyses available for comparison, the presence of unknown amino-acids outside of this group seems improbable Whether the losses of isolating those of this group arise from the presence of new, as yet unrecognized mono-amino-acids, or from the unavoidable losses connected with the methods of isolation, can not at present be stated



# THE PARTIAL HYDROLYSIS OF PROTEINS

## III ON FIBRIN PROTOALBUMOSE

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(Received for publication, June 17, 1911 )

The differences in solubility and in general properties between proto- and hetero-albumose are very striking. The existence of the two bodies as distinct substances has been recognized by most investigators. The statements regarding the differences in the chemical composition of the two albumoses were at times contradictory. Pick was the first to record very definite distinctions in the number and in the proportion of different amino-acids present in the molecule of the two substances. Later, Levene found that by employing larger quantities of material for the experiments, the same amino-acids could be obtained from the hetero- and the proto-albumose. Still later Birchard, working in Siegfried's laboratory, recorded the absence of glutamic acid in the molecule of proto-albumose. The results published by Levene referred only to the character of the amino-acids present in the molecule and not to their relative proportion. The methods of analysis have improved considerably since the time of that publication and it was decided to repeat the hydrolysis.

Attempts were made to obtain the albumose in as high a degree of purity as present methods of preparation permit. It was possible at this time also to isolate the same amino-acids on hydrolysis of proto- as on the hydrolysis of the hetero-albumose. However, there were very striking distinctions in the proportions of some of them.

The most striking difference was found in the proportion of glutamic acid. While the hetero-albumose contained 9.51 per cent of this acid, only 0.63 per cent were separated out from the products of hydrolysis of the proto-albumose. The possibility is not excluded that the small quantity of the acid belonged to

some impurity. However, the result of the present hydrolysis confirms the view of Birchard, that the principal distinction of the albumoses lies in their content of glutaminic acid.

The differences in the leucine group (leucine, isoleucine, and valine) are also marked. The low content of the proto- in valine is striking compared with the valine content of hetero-albumose. The fact that more glycocoll was found in the proto-albumose, cannot be taken into consideration in comparing the two, because the methods used in order to isolate the glycocoll were not the same in each case. By the ordinary esterification practically none would have been obtained from the proto-albumose.

The proportion of amid nitrogen was found higher in the hetero-albumose. This finding is not unexpected on the ground of the high glutaminic acid content of this substance.

The generally accepted view, that the hetero-albumose is characterized by a higher content of hexone bases could not be corroborated by the present investigation. The difference of the content in the total hexone bases of the two albumoses was found very insignificant. Only in regard to the proportion of histidine the difference was marked, showing 3.90 per cent in the hetero- and 2.77 per cent in the proto-albumose.

## EXPERIMENTAL PART

### *Preparation*

The proto-albumose was prepared by a modification of Pick's method and was essentially the same as described by one of us in a former paper<sup>1</sup>. It was there shown that from any preparation of proto-albumose prepared according to Pick, a fraction could be separated which in its physical properties corresponded to hetero-albumose. The method consists in a suitable application of Siegfried's carbamino reaction which has also been applied to the separation of peptones and amino acids<sup>2</sup>. The details are as follows.

<sup>1</sup> F. J. Birchard *Ein Beitrag zur Kenn'nis der Protoalbumose des Fibrins*, Dissertation, Leipzig, 1909.

<sup>2</sup> M. Siegfried *Zeitschr. f. physiol. chem.*, **xliv**, p. 85, 1910, *Ber. d. d. chem. Ges.*, **xxix**, p. 397, 1910. See also *Ergeb. d. Physiol.*, **ix**, pp. 334-350, 1910.

The primary albumoses were precipitated from a ten per cent solution of Witte's peptone in the manner described in our second communication.<sup>1</sup> The sulphate in the alcoholic solution of the proto-albumose was removed by barium hydrate, and the slight excess of barium by ammonium carbonate. The filtrate was concentrated in a vacuum to a small volume, alcohol being added from time to time to prevent foaming. The thick syrup remaining was precipitated by alcohol and dried in a desiccator over sulphuric acid.

One hundred and eighty grams of the dried, finely powdered albumose were dissolved in 8 liters of a cold saturated solution of barium hydrate. The vessel containing the mixture was kept surrounded by ice and care was taken that the temperature never rose above 4° C. Carbon dioxide was then passed through until the solution was almost neutral, the mixture being stirred continuously during the whole process. 2800 cc of a cold saturated solution of barium hydrate were further added and the whole allowed to stand until the precipitate had completely settled. It was found convenient to place the vessel in the refrigerator over night, when the greatest part of the clear solution could be easily filtered off. The precipitate was washed on a Buchner funnel with 600 cc of a half-saturated solution of barium hydrate cooled to 0°. Great care was taken to keep the temperature of the precipitate as low as possible during the filtering and washing. The filtrate and wash-water were united, ammonium carbonate added to remove the barium, and the whole heated for a short time on the water-bath. The filtrate was then concentrated in a vacuum to a thick syrup, taken up in dilute alcohol, and finally precipitated in a large excess of alcohol and dried over sulphuric acid. The albumose was obtained finally as a white powder, easily soluble in water and in 66 per cent alcohol.

**ANALYSIS** The preparation contained 1.5 per cent of ash. The analytical figures are reckoned on ash-free substance.

0.2200 gm substance    26.10 cc  $\frac{N}{10}$  acid    16.46 per cent N

0.2805 gm substance    33.20 cc  $\frac{N}{10}$  acid    16.53 per cent N

0.1324 gm substance    0.2599 gm CO<sub>2</sub>    0.0359 gm H<sub>2</sub>O  
C = 53.60 per cent    H = 7.20 per cent

0.1322 gm substance    0.2598 gm CO<sub>2</sub>    0.0313 gm H<sub>2</sub>O  
C = 53.59 per cent    H = 6.83 per cent

**AMINO NITROGEN** The amino nitrogen was determined according to the method of Van Slyke.

2 gm albumose were dissolved in 50 cc water

10 cc solution    40.2  $\frac{N}{10}$  acid = 0.5628 gm N

10 cc solution    3.25 cc amino N, at 19°, 757 mm = 0.0037 gm amino N

Amino N = 6.58 per cent of total N

<sup>1</sup> P. A. Levene, D. D. Van Slyke, and F. J. Birchard. This Journal, viii, p. 269, 1910.

<sup>2</sup> D. D. Van Slyke. *Proc Soc Exp Biol and Med*, Dec 15, 1909, *Ber d chem Ges*, xliii, p. 3170, 1910.

AMMONIA NITROGEN The albumose contained practically no free ammonia 5 gms of substance, according to Folin's method, neutralized 1.20 cc  $\frac{N}{10}$  acid

### Hydrolysis

One hundred and thirty-two grams of proto-albumose were hydrolyzed with 20 per cent hydrochloric acid for sixteen hours. The solution was concentrated to a small volume in vacuum until it was of a thick syrupy consistence, then saturated with hydrochloric acid gas, a small crystal of glutaminic hydrochloride added, and allowed to stand in the refrigerator for ten days. The crystals were filtered off over asbestos, washed with cold concentrated hydrochloric acid, dissolved in water and a small amount of barium present quantitatively removed with sulphuric acid. The solution (30 cc) was then saturated with hydrochloric acid and allowed to stand a week in the refrigerator. The crystals which separated were not, however, pure glutaminic hydrochloride, the analysis indicating the possible presence of leucine. It was again recrystallized (volume 90 cc) when 1.0 gram of pure glutaminic hydrochloride was obtained.

ANALYSIS 0.1583 gm substance 0.1233 gm silver chloride  
0.1063 gm substance 14.2 cc N at 16°, 762 mm

	Calculated for $C_6H_9NO_4 \cdot HCl$	Found
Cl	19.31 per cent	19.24 per cent
N	7.63 per cent	7.76 per cent

### Esterification

The amino-acids in the mother liquors were esterified by Fischer's method. Two crops of esters were freed by the barium hydrate method.<sup>1</sup> The following fractions were obtained on distillation.

FRACTION	TEMPERATURE	PRESSURE	YIELD OF ESTERS
		mm	grams
I	to 70°	12	40.7
II	to 70°	0.06	28.6
III	to 95°	0.16	15.2
IV	Residue		38.8

<sup>1</sup> Levene and Van Slyke. *This Journal*, vi, p 391, 1909

The last fraction, usually containing the aspartic acid, glutamic acid, and phenylalanine, was not distilled, but worked up in the way suggested by Osborne<sup>1</sup>

*Fraction I* This consisted chiefly of alcohol. It yielded only 2.4 grams of amino-acids, from which 0.9 gram of alanine was obtained by recrystallization.

ANALYSIS 0.0798 gm substance 21.95 cc N at 20°, 762 mm (Nitrous acid method)  
0.1432 gm substance 0.2165 gm CO<sub>2</sub> 0.1036 gm H<sub>2</sub>O

	Calculated for C <sub>3</sub> H <sub>7</sub> O <sub>2</sub> N	Found
N	15.73	15.69
C	40.42	41.20
H	7.93	8.04

The mother liquors yielded 0.4 gram of a mixture of alanine and glycocoll (17.10 per cent of nitrogen). The mother liquors from this crop were added to the most soluble amino-acids of fraction II and worked up for proline.

*Fraction II* As the albumose contained very little valine, this fraction yielded all of its leucine in pure form by direct crystallization, the amount being 4.76 grams.

ANALYSIS 0.1050 gm substance 20.4 cc N at 20°, 762 mm (Nitrous acid method)  
0.0816 gm substance 0.3660 gm CO<sub>2</sub> 0.1585 gm H<sub>2</sub>O

	Calculated for C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub>	Found
C	54.92	54.96
H	9.99	9.76
N	10.69	10.87

ROTATION IN 20 PER CENT HCl 0.2259 gm substance 4.272 gm solution  
rotation in 1 dm tube, +1.16°

$$[\alpha]_D^{20} = +19.93^\circ$$

From the rotation, the leucine consisted of 80.35 *L*-leucine and 19.7 per cent *D*-isoleucine.<sup>2</sup>

The mother liquors yielded a mixture of valine and alanine (nitrogen content, 13.17 per cent) weighing 2.33 grams. One

<sup>1</sup> *Amer Jour of Physiol*, xxvi, p 212, 1910

<sup>2</sup> Levene and Van Slyke *This Journal*, vi, p 400, 1909



gram was used for separation of the two by precipitation of the alanine with concentrated phosphotungstic acid<sup>1</sup> It yielded 0.20 gram of alanine and 0.71 gram of valine

ANALYSIS OF ALANINE 0.0647 gm substance 17.4 cc N at 18.5°, 768 mm (Nitrous acid method)

	Calculated for $C_3H_7O_2N$	Found
N	15.73	15.58

ANALYSIS OF VALINE 0.0922 gm substance 19.6 cc N at 21°, 768 mm (Nitrous acid method)

	Calculated for $C_6H_{11}O_2N$	Found
N	11.96	12.18

ROTATION OF VALINE IN 20 PER CENT HCl 0.1840 gm substance 4.453 gm solution concentration, 4.13 per cent rotation in 1 dm tube, +1.14°  
 $[\alpha]_D^{20} = +25.1^\circ$

The mother liquors from the above fraction were added to those from the most soluble portions of I and III to be extracted for proline

*Fraction III* This fraction yielded only leucine, isoleucine, and proline The proline was extracted with absolute alcohol and combined with the extracts of I and II

The alcohol-insoluble amino-acids yielded by recrystallization 0.41 gram of *L*-leucine nearly free from isoleucine

ANALYSIS 0.0989 gm substance, 18.9 cc N at 18.5°, 756 mm

	Calculated for $C_6H_{13}O_2N$	Found
N	10.69	10.77

ROTATION 0.2143 gm substance, 4.300 gm solution in 20 per cent HCl  
 Rotation in 1 dm tube, +0.91°  
 $[\alpha]_D^{20} = +16.6^\circ$

By further crystallization two other crops were obtained containing increasing amounts of isoleucine The first weighed 3.96 grams of the following composition and rotation

ANALYSIS 0.1321 gm substance, 25.00 cc N at 21°, 762 mm  
 0.1533 gm substance, 0.3076 gm CO<sub>2</sub>, 0.1340 gm H<sub>2</sub>O

	Calculated for $C_6H_{13}O_2N$	Found
N	10.69	10.75
C	54.92	54.73
H	9.99	9.78

<sup>1</sup> Levene and Van Slyke Not yet published

ROTATION IN 20 PER CENT HC 0 2384 gm substance, 4 399 gm solution, concentration, 6 56 per cent, rotation in 1 dm tube, + 1 46,<sup>o</sup>  
 $[\alpha]_D^{20} = + 20\ 23^{\circ}$

From the rotation, the substance contained 78 7 per cent *l*-leucine and 21 3 per cent *d*-isoleucine

A third crop of leucine weighing 1 73 grams was obtained, still richer in isoleucine

ANALYSIS 0 1186 gm substance, 21 85 cc N at 20°, 762 mm, (Nitrous acid method)

	Calculated for $C_6H_{13}O_2N$	Found
N	10 69	10 52

ROTATION IN 20 PER CENT HCl 0 1531 gm substance, 4 266 gm solution, concentration 3 59 per cent, rotation in 1 dm tube, + 1 10<sup>o</sup>  
 $[\alpha]_D^{20} = + 27\ 85^{\circ}$

From the rotation, this crop contained 43 8 per cent *l*-leucine and 56 2 per cent *d*-isoleucine

*Phenylalanine* The phenylalanine ester was removed from the other esters of Fraction IV in the usual manner by extracting with ether Nearly all of the black coloring matter of the mixture went with the phenylalanine ester into the ether The phenylalanine ester was not treated with hydrochloric acid, but was saponified by boiling with water The black impurities proved insoluble in the water and stuck to the sides of the flask when the solution was poured out The first crop of phenylalanine, crystallizing as the result of concentration and addition of alcohol, contained 8 87 per cent of nitrogen instead of 8 49 After one recrystallization it showed the following composition

ANALYSIS 0 0951 gm substance, 13 9 cc N at 20°, 770 mm

	Calculated for $C_9H_{11}O_2N$	Found
N	8 49	8 93

The mother liquors yielded 2 14 grams of crude phenylalanine containing 7 8 per cent of nitrogen by the nitrous acid method After one recrystallization the substance gave the following analysis

0 0903 gm substance, 13 3 cc N at 15°, 750 mm

	Calculated for $C_9H_{11}O_2N$	Found
N	8 49	8 47

From an esterification, described below, of the residues left after extraction of the esters yielding the above amino-acids, 0.40 gram more of phenylalanine was obtained, making the total yield 5.74 grams

*Aspartic Acid* The esters of Fraction IV left in water solution after extracting the phenylalanine ester were saponified with barium hydrate in the usual manner, and the solution of the amino-acids was concentrated and saturated with hydrochloric acid in an attempt to obtain glutaminic acid. No glutaminic hydrochloride crystallized even after several days standing in the refrigerator. The solution was therefore freed from hydrochloric acid by evaporation, followed by treatment with silver sulphate and barium hydrate, and was then concentrated and an attempt made to crystallize aspartic acid after addition of alcohol to the solution. A crop of amino-acids weighing 6.75 grams crystallized out, but it contained 9.61 per cent of nitrogen instead of 10.54 per cent, and also gave an appreciable Millon reaction. In order to ascertain the amount of aspartic acid present in the crude substance, 0.1996 gram was dissolved in water and titrated with rosolic acid as indicator. 8.70 cc of  $\frac{N}{10}$  sodium hydrate were neutralized, indicating that the crude substance contained 58.2 per cent or 3.92 grams of aspartic acid. For purification it was converted into the copper salt. This crystallized after some time in pure condition. It was dried at 110°

ANALYSIS 0.2456 gm substance, 0.0998 gm CuO

Cu	Calculated for	Found
	$\text{CuC}_4\text{H}_5\text{O}_4\text{N}$	
	32.67	32.49

*Proline* The alcoholic extracts of the amino-acids from fractions I, II, and III were united, concentrated, and taken up as usual in cold absolute alcohol. The alcohol was removed from the solution by concentration, and the residue dissolved in water. The solution was brought to 250 cc volume and 10 cc portions taken for determination of amino and total nitrogen. The amino determinations gave 17.65 and 17.85 cc of nitrogen gas at 18.5 and 765 mm, the average being equivalent to 0.256 gram of amino nitrogen in the entire solution. The portions for Kjeldahl determinations neutralized 28.48 and 28.58 cc of  $\frac{N}{10}$  acid, equivalent to 1.000 gram of nitrogen. The difference between the two

nitrogen determinations gives 0.744 gram of proline nitrogen, equivalent to 6 grams of proline, or 4.64 per cent of the albumose. The esterification of the residues, described below, yielded 0.44 gram more of proline, bringing the total up to 6.55 grams or 4.96 per cent.

The proline in 200 cc. of the solution used for the above nitrogen determinations was racemized and converted into the copper salt, of which 3.31 grams of the following composition was obtained after recrystallization:

0.1926 gm. substance, 1.8 cc. N at 19° 752 mm. by the nitrous acid method  
 0.4572 gm. substance, 0.0479 gm. loss at 110°  
 0.4572 gm. substance, 13.82 cc.  $\frac{N}{V}$  sulphocyanate (Volhard copper titration)

	Calculated for $Cu(C_5H_7O_2N)_2 \cdot 2H_2O$	Found
H <sub>2</sub> O	10.99	10.48
Cu	19.40	19.23
NH <sub>2</sub>	0.00	0.52

### *Esterification of the Residues*

We have previously observed that glycocoll and serine esters appear to escape extraction by ether<sup>1</sup> to a large extent, as more of these amino-acids could be obtained from the unextracted residues than from the esters. Consequently we re-esterified the residues left after extracting the esters above described, and freed the esters from this last portion by means of sodium ethylate, avoiding chance of loss from the difficulty of extracting the more soluble esters from water.

The residues from the main esterification were combined with the mother liquors from Fraction IV, which it was thought might contain some serine, and the mixture was re-hydrolyzed in order to split any anhydrides which might have been formed by the esters. The hexone bases were then precipitated by phosphotungstic acid in the presence of hydrochloric acid. The greater portion of the phosphotungstic acid in the filtrate was removed by extraction with ether. The hydrochloric acid was then removed as completely as possible by concentrating the solution under

<sup>1</sup>P. A. Levene, D. D. Van Slyke and F. J. Birchard. *This Journal*, viii, p. 269, 1910.

diminished pressure, and the small amount of phosphotungstic acid not removed by the ether was precipitated with barium hydrate solution. The excess of barium was then removed by means of carbon dioxide and sulphuric acid. The above procedure was adopted in place of the more commonly used precipitation of the bases in the presence of sulphuric acid followed by removal of phosphotungstic and sulphuric acids together with barium, because we have found that the bulky precipitate of phosphotungstate and sulphate of barium adsorbs a considerable proportion of the amino-acids, and that these acids cannot be regained by mere washing.

The solution obtained was esterified in the usual manner, the esters were freed with sodium ethylate, and distilled in two fractions, the temperature of the vapors running up to  $90^{\circ}$  in the first fraction at 0.5 mm, that of the vapors of the second fraction up to  $115^{\circ}$ . Extraction of the amino-acids from the first fraction with alcohol yielded a solution containing 0.0820 gram of nitrogen, of which 0.0285 gram was amino nitrogen, the non-amino nitrogen corresponding to 0.44 gram of proline. The rest of this fraction consisted almost entirely of glycocoll of which 1.88 grams crystallized in pure condition when the solution of amino-acids insoluble in alcohol was concentrated and treated with alcohol.

ANALYSIS 0.0965 gm substance, 32.5 cc N at  $20^{\circ}$ , 762 mm (Nitrous acid method)

	Calculated for $C_5H_9NO$	Found
N	18.69	19.22

ROTATION IN WATER SOLUTION Solution of 3.30 per cent concentration in 1 dm tube. No optical activity whatever.

The nitrogen content is characteristic of glycocoll, which always gives off a trace of other gas when treated with nitrous acid, so that the results come out about 0.5 per cent high.

From the higher boiling fraction of esters, which weighed 9 grams nothing crystalline could be obtained except 0.4 gram of phenylalanine.

ANALYSIS 0.0620 gm substance, 9.00 cc N at  $20^{\circ}$ , 776 mm (Nitrous acid method)

	Calculated for $C_9H_{11}O_2N$	Found
N	8.49	8.44

*Tyrosine*

A solution containing 40.2 grams of albumose was hydrolyzed with 20 per cent hydrochloric acid. The acid was removed by evaporation, followed by treatment with silver sulphate and barium hydrate. From the concentrated solution 1.83 grams of tyrosine crystallized.

ANALYSIS 0.1257 gm substance, 17.1 cc N at 18°, 764 mm  
0.1946 gm substance, 0.4260 gm CO<sub>2</sub>, 0.1104 gm H<sub>2</sub>O

	Calculated for C <sub>9</sub> H <sub>11</sub> O <sub>3</sub> N	Found
N	7.73	7.86
C	59.67	59.70
H	6.08	6.34

*Determination of the Hexone Bases and Nitrogen Distribution by the Method of Van Slyke*

Seven grams of albumose were hydrolyzed with 75 cc of 20 per cent hydrochloric acid. After 6, 9, 16, and 24 hours samples of 1 cc were withdrawn, diluted to 10 cc and used for determinations of amino nitrogen. The results were the same after 16 and 24 hours indicating that hydrolysis was complete in 16 hours. The greater portion of the hydrochloric acid was removed by concentrating the solution under diminished pressure, and the residue was dissolved and brought to 250 cc. Ten cubic centimeter portions were taken for Kjeldahl determinations. They neutralized 25.63 and 25.59 cc of decinormal acid. For the determinations duplicate portions of 100 cc, each containing 0.358 gram of nitrogen, were taken. The determinations were carried out as described in the accompanying paper by Van Slyke.

*Amid Nitrogen* The ammonia from each sample neutralized 12.1 cc of  $\frac{N}{10}$  sulphuric acid, indicating 0.0169 gm of nitrogen or 4.73 per cent.

*Amino Nitrogen in the Bases* One-fifth of the solution obtained by dissolving the phosphotungstate precipitate was used for determination of amino nitrogen. The amounts of nitrogen given off were

I 18.9 cc at 20°, 760 mm, II 19.0 cc at 20°, 760 mm

*Arginine Determination* One-half of the solution of the bases was used for each arginine determination. The amounts of  $\frac{N}{10}$  acid neutralized were

I 9.40 cc, II 9.60 cc

*Total Nitrogen of the Bases* The same solutions as used for arginine were employed for determination of total nitrogen. They neutralized by the Kjeldahl method

I 37.1 cc, II 37.9 cc of  $\frac{N}{10}$  acid

*Cystine in the Bases* One-fifth of the solution in each case was used for determination of cystine sulphur by the Benedict-Denis method. Each yielded 6 mg of barium sulphate.

*Total Nitrogen in the Filtrate from the Bases* The filtrates were brought to 150 cc each and 25 cc portions used for Kjeldahl determinations. There were neutralized

I 26.08 cc, II 25.80 cc of  $\frac{N}{10}$  acid

*Amino Nitrogen in the Filtrate from the Bases* Ten cc portions were utilized for determination of amino nitrogen. They gave off

I 22.7-22.9, average 22.8 cc of nitrogen at 20°, 760 mm

II 22.4-22.45, average 22.42 cc of nitrogen at 20°, 760 mm

*Melanin* The melanin adsorbed by the lime used in boiling off the amid nitrogen was determined by the Kjeldahl method. It neutralized

I 10.15 cc, II 10.88 cc of  $\frac{N}{10}$  acid

The results are summarized in the following table.

TABLE I  
*Nitrogen Distribution of Proto-Albumose*

	I	II	AVERAGE
Amid nitrogen	4.73	4.73	4.73
Arginine nitrogen	14.71	15.03	14.89
Histidine nitrogen	4.06	5.00	4.53
Lysine nitrogen	9.50	9.70	9.60
Cystine nitrogen	0.50	0.50	0.50
Amino nitrogen (filtrate)	54.3	53.5	53.9
Non-amino nitrogen in filtrate	6.8	7.1	6.95
Melanin nitrogen	3.97	4.25	4.11

The arginine nitrogen corresponds to 7.72 per cent of arginine as the amino-acid in the albumose, the histidine to 2.77, the lysine to 8.40

#### *Determination of the Hexone Bases and Nitrogen Distribution in Hetero-Albumose*

In our previous paper on hetero-albumose we published the results of a determination of the hexone bases and nitrogen distribution by the method as used at that time. We have since

found that loss could occur through incomplete solution of the hexone base precipitate. As the nitrogen in the filtrate was at that time determined by difference, such loss would cause too high results for the filtrate, especially in the non amino nitrogen. We have repeated, therefore, this determination in duplicate by the present method, and find that a considerable loss did undoubtedly occur in the first determination. Our present results follow.

Ten grams of air dried hetero-albumose were hydrolyzed in a manner above described, and the solution brought to 250 cc. Ten cc portions used for Kjeldahl determinations neutralized 43.05 cc each of  $\frac{N}{10}$  acid. Duplicate determinations of the nitrogen distribution were made on portions of 75 cc each containing 0.452 gm of nitrogen.

*Amid Nitrogen* The ammonia from each sample neutralized 20.5 cc of  $\frac{N}{10}$  sulphuric acid.

*Amino Nitrogen in the Bases* One-fifth of the solution obtained by dissolving the phosphotungstic precipitate was used for determination of amino nitrogen. The amounts of nitrogen given off were

I 26.35 cc, II 26.35 cc at 16°, 570 mm

*Arginine Determination* One-half of the solution of the bases was used for each arginine determination. The amounts of  $\frac{N}{10}$  acid neutralized were

I 11.25 cc, II 11.60 cc

*Total Nitrogen of the Bases* The same solutions as used for arginine were employed for determination of total nitrogen. The amounts of  $\frac{N}{10}$  acid neutralized were

I 51.50 cc, II 51.14 cc

*Cystine in the Bases* One-fifth of the solution was used for each determination. The yields of barium sulphate were

I 14.8 mg, II 13.2 mg

*Total Nitrogen in the Filtrate of the Bases* The filtrates were brought to 150 cc and 25 cc proportions were used for Kjeldahl determinations. The amounts of  $\frac{N}{10}$  acid neutralized were

I 31.1-31.4 cc, II 31.9-32.0 cc

*Amino Nitrogen in the Filtrate of the Bases* Ten cc portions were utilized for determination of amino nitrogen. They gave off

I 29.15-29.23 cc, II 29.95-30.10 cc of nitrogen at 21°, 750 mm

*Melanin* The amounts of  $\frac{N}{10}$  acid neutralized were

I 9.05 cc, II 8.30 cc



The results are summarized in the following table

TABLE II  
*Nitrogen Distribution of Hetero-Albumose*

	I	II	AVERAGE
Amid nitrogen	6 37	6 37	6 37
Arginine nitrogen	13 93	14 36	14 15
Histidine nitrogen	6 53	6 37	6 45
Lysine nitrogen	10 17	10 12	10 30
Cystine nitrogen	0 97	0 86	0 91
Amino nitrogen in filtrate	54 1	55 7	54 9
Non-amino nitrogen in filtrate	4 0	3 7	3 85
Melanin nitrogen	2 8	2 7	2 75

The arginine nitrogen corresponds to 7.3 per cent of arginine as the amino-acid in the albumose, the histidine to 3.9 per cent, the lysine to 8.9 per cent

In the following table are compared the mean results for hetero- and proto-albumoses and for fibrin, the native protein from which they originate, the experimental data for the latter being given in the accompanying paper on the method

TABLE III  
*Comparison of Hetero-Albumose, Proto-Albumose and Fibrin*

	HETERO- ALBUMOSE	PROTO- ALBUMOSE	FIBRIN
Amid nitrogen	6 37	4 73	8 31
Arginine nitrogen	14 15	14 89	13 64
Histidine nitrogen	6 45	4 53	3 96
Lysine nitrogen	10 30	9 60	11 40
Cystine nitrogen	0 91	0 50	0 43
Amino nitrogen in the filtrate	54 9	53 9	55 4
Non-amino nitrogen in the filtrate	3 85	6 95	3 90
Melanin nitrogen	2 75	4 11	3 15

The differences in the cystine are of no significance because the cystine is partially destroyed during the hydrolysis

In the following table are summarized the results of the complete hydrolysis of hetero- and proto-albumose. The figures indicate grams of amino-acid from 100 grams of albumose

TABLE IV

	HETERO-ALBUMOSE	PROTO-ALBUMOSE
Glutamic acid	9.51	0.63
Leucine	3.05	5.79
Isoleucine	2.96	1.62
Valine	3.54	0.76
Alanine	3.39	2.50
Valine-alanine mixture	1.86	0.00
Proline	4.27	4.96
Phenylalanine	2.45	4.35
Aspartic acid	4.73	2.98
Glycocoll	0.15	1.44
Tyrosine	3.48	4.58
Arginine	7.30	7.72
Histidine	3.90	2.77
Lysine	8.90	8.40
Cystine	1.36	0.68
Ammonia	1.28	0.92



## OXIDATION OF THE AMINO ACIDS II ALANINE AND TYROSINE

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(Received for publication, June 19, 1911)

In a previous communication<sup>1</sup> I have given an account of the oxidation of glycocoll and cystine with alkaline potassium permanganate, the work has been continued along the same lines and in the present paper are recorded the results obtained by the oxidation of two other of the more common amino-acids—alanine and tyrosine

### *Oxidation of Alanine with Alkaline Potassium Permanganate*

Two grams of Kahlbaum's alanine dissolved in 50 cc of water were oxidized with 500 cc of a solution containing 10.8 grams of potassium permanganate and 37.0 grams of potassium hydroxide (30 molecules). Vigorous oxidation took place at once and the temperature of the mixture rose to 37° C. After standing for forty-eight hours at room temperature complete decolorization had taken place, the products of oxidation were then isolated and quantitatively determined by the methods previously described.

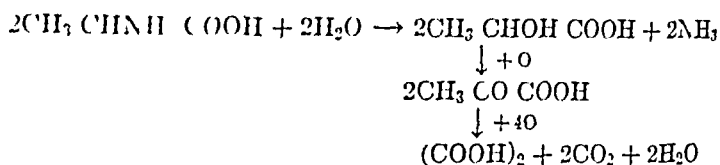
From the above mentioned 2 grams of alanine I obtained 1.45 grams of carbon dioxide, 0.37 gram of ammonia, 1.9 grams of oxalic acid melting at 101° and volatile acids equivalent to 10 cc of decinormal sodium hydroxide.

The volatile acid fraction was found on the application of qualitative tests to consist of a mixture of acetic and nitric acids.

Regarding the intermediate products it would seem most probable that the greater part of the alanine on oxidation with alkaline

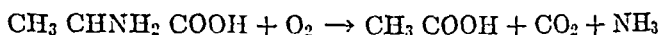
<sup>1</sup> This *Journal*, ix, p 365, 1911

permanganate would first undergo a transformation into lactic acid with subsequent oxidation to pyruvic, oxalic and carbonic acids



However in a number of experiments in which alanine was oxidized under various conditions with an insufficient amount of alkaline permanganate solution I was unable to obtain qualitative proof of the presence of pyruvic acid

The isolation of a minute quantity of fatty acid would seem to indicate that a small portion of the alanine is transferred directly into acetic and carbonic acids



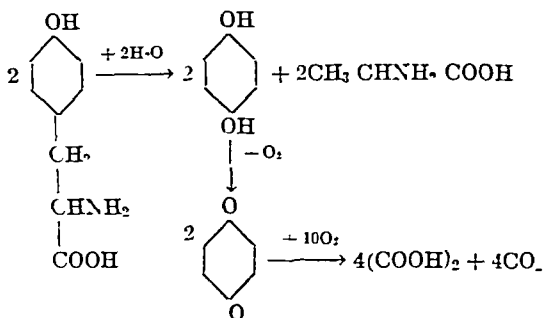
#### *Oxidation of Tyrosine with Alkaline Potassium Permanganate*

The tyrosine used in this work was prepared from silk by hydrolysis with 20 per cent sulphuric acid. To 2 grams of tyrosine suspended in 50 cc of water were added 600 cc of a mixture containing 19.0 grams of potassium hydrate (30 molecules) and 17.0 grams of potassium permanganate. Vigorous oxidation began at once and the mixture was allowed to stand at room temperature for two days, as a small amount of the permanganate still remained unreduced. The solution was then heated for an hour on the boiling water bath, ammonia, carbon dioxide, oxalic acid and volatile acids were determined by the methods previously described. In this way I obtained from the above mentioned 2.0 grams of tyrosine 0.18 gram of ammonia, 2.02 grams of carbon dioxide, 2.1 grams of oxalic acid, volatile acids equivalent to 16.00 cc of decinormal sodium hydrate. The volatile acids were found to consist of 0.03 gram of acetic acid, and some nitric acid, in the early part of the distillation a small amount of a white crystalline body was driven over, this substance which is probably *p*-oxybenzoic acid was formed in such minute quantities that a positive identification was impossible.

The oxalic acid obtained was identified by its melting point,  $99^{\circ}\text{C}$ , rising after desiccation over sulphuric acid to  $189^{\circ}$ , and by the properties of its calcium salt

From these results it is apparent that in the oxidation of tyrosine by alkaline permanganate approximately four carbon atoms are utilized in the formation of carbon dioxide while the remaining five carbon atoms go to the production of oxalic, acetic, and probably *p*-oxy benzoic acids

The ease with which the benzene ring is broken would seem to point to the intermediate formation of quinone compounds with the tearing apart of the side chain and benzene ring



In order to obtain proof of this theory I have oxidized hydroquinone by exactly the method used for tyrosine

Two grams of hydroquinone suspended in 50 cc of water were treated with 600 cc of a mixture containing 32.2 grams of potassium hydrate (30 molecules) and 18.6 grams of potassium permanganate, after standing for two days at room temperature decolorization of the permanganate was almost complete, the mixture was then heated for half an hour on the boiling water bath to obtain complete reduction. In this way I obtained 2.56 grams of oxalic acid, 2.52 grams of carbon dioxide and on distilling after acidifying the solution a small amount of a white crystalline acid body condensed in the receiver which had the same properties as the substance obtained in a similar way on the oxidation of tyrosine, no volatile fatty acids could be detected in the distillate

## SUMMARY

1. The products produced by the oxidation of alanine with alkaline potassium permanganate were shown to be ammonia, carbon dioxide, oxalic, acetic and nitric acids

2 On oxidation of tyrosine with alkaline potassium permanganate the oxidation products were found to consist of ammonia, carbon dioxide, oxalic, acetic and nitric acids, together with traces of an acid which was thought to be *p*-oxybenzoic acid but which owing to the minute amounts available was not positively identified

# THE PHOSPHORUS ASSIMILATION OF *ASPERGILLUS NIGER*

By ARTHUR W. DOX

(From the Chemical Section of the Iowa Agricultural Experiment Station )

(Received for publication, June 22, 1911 )

In his classic researches on the nutritive requirements of lower fungi, Raulin<sup>1</sup> made the observation more than forty years ago that the presence of a phosphate in the medium is necessary for the support of life of *Aspergillus niger*. The amount required was indeed small, but when this was omitted from the culture medium, the mold failed to develop. Since that time a number of different culture media have been formulated by other investigators, but all contain phosphorus in some form. In the simplest of the synthetic media described, the phosphorus is added in the form of an ammonium or potassium salt of phosphoric acid, while the more complex media, such as infusions and decoctions of various plant and animal products, may contain part or all of the phosphorus in organic combination.

The carbon and nitrogen assimilation of molds have been made the subject of many interesting investigations.<sup>2</sup> Growth phenomena have been studied by varying the carbonaceous or nitrogenous constituent of the medium, and in this way many interesting observations have been made. The common saprophytic molds were found to have the power of utilizing a surprisingly large variety of chemical substances as sources of carbon and nitrogen.

<sup>1</sup>*Ann sc nat* (5) bot, vi, p 93

Naegeli *Bot Mitth*, iii, p 395, Reinke *Bot Zeitung*, xli, p 551, Duclaux *Compt rend soc biol*, xxxvii, p 91, Diakonow *Ber bot Gesellsch*, v, p 380, Puriewitsch *ibid*, xiii, p 342, Emmerling *Ber d d chem Ges*, xxxv, p 2289, Czapek *Hofmeister's Beiträge*, i, p 542, iii, p 47, Abderhalden *Zeitschr f physiol Chem*, xlvii, p 394, Hasselbring *Bot Gazette*, xlv, p 176, Ritter *Ber bot Ges*, xxvii, p 582, Pringsheim *Chem Zentralbl*, 1910, ii p 101



Of the other elements necessary for the support of plant life, phosphorus is perhaps the only one which can be varied to any extent in the culture medium. It can be supplied in a variety of forms, depending upon valence, state of hydration, and the property of forming ester-like combinations with organic radicles. The cultures described in this paper were made for the purpose of determining the availability of different compounds of phosphorus as food material for lower fungi.

Raulin's medium, from which the prescribed ammonium phosphate was omitted, was used throughout these experiments, and an equivalent amount of phosphorus in some other form substituted. In order to avoid any hydrolysis of the phosphorus compound that might result from sterilization of the medium, the latter was sterilized separately, and the source of phosphorus to be tested was afterwards added under aseptic conditions. The resulting media were then inoculated from a pure culture of *Aspergillus niger* and kept in the dark at room temperature.

Various forms of inorganic phosphorus were first tried. The salts were carefully purified and analyzed, so that the exact amount of phosphorus corresponding to that contained in Raulin's medium could be used. The cultural data are given in the following table.

SUBSTANCE	FORMULA	PER CENT P	AMOUNT ADDED	GROWTH IN ONE WEEK
			<i>per cent</i>	
Sodium orthophosphate (anhydrous)	$\text{Na}_2\text{HPO}_4$	21.8	0.0431	Excellent
Sodium pyrophosphate	$\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$	6.95	0.1354	Excellent
Sodium metaphosphate	$\text{NaPO}_3$	30.4	0.0309	Excellent
Sodium hypophosphite	$\text{NaH}_2\text{PO}_2 \cdot \text{H}_2\text{O}$	29.2	0.0322	Germination only
Sodium phosphite	$\text{Na}_2\text{HPO}_3 \cdot 5\text{H}_2\text{O}$	14.4	0.0653	Nil

Pyro- and meta-phosphates when kept in solution tend to combine with water and revert to the orthophosphate. Whether or not this occurred to any extent in the cultures was not determined positively. From the fact that the cultures on these two substances germinated and developed as rapidly as that on the orthophosphate, we are inclined to suppose that these salts must have been utilized directly. Sodium hypophosphite and sodium phosphite are evidently not available as sources of phosphorus. This failure to support life was not due to a direct toxic action, as

was shown by separate cultures in which orthophosphate was present in addition to phosphite and hypophosphite respectively. When the orthophosphate was present in the usual amount, a concentration of 3 per cent of sodium hypophosphite was necessary to suppress completely the growth of mold, while this concentration of sodium phosphite did not entirely check growth. The toxic effect in these two instances was caused as much perhaps by the sodium ion as by the reduced phosphorus. While the mold grows in ammonium phosphate or ammonium acid phosphate in concentrations up to 30 per cent, a concentration of sodium phosphate (anhydrous salt) of 2.3 per cent entirely inhibits growth.

The effect of increasing concentration of these salts is of course a gradual one. With the optimum amount in the medium, the mold germinates within twenty-four hours and rapidly develops a thick mycelium which is usually full of wrinkles and corrugations, evidently for the purpose of presenting a greater surface for absorption of the nutritive medium. At the same time the development of black spores is quite rapid. An increase in the amount of a given salt in the medium beyond the optimum tends to retard germination and render the growth of mycelium less dense, while the spore formation is delayed or even suppressed. Beyond a certain concentration, germination and development may be entirely inhibited.

Several substances containing phosphorus in organic combination were also tested as sources of this element. In all of these substances the phosphorus is in its higher state of oxidation, and, as will be seen from the following table, is readily utilized.

SUBSTANCE	PER CENT P	AMOUNT ADDED	GROWTH IN ONE WEEK
		<i>per cent</i>	
Phytin	21.60	0.0435	Excellent
Sodium glycerinophosphate	13.11	0.0717	Excellent
Sodium nucleinate	7.92	0.1187	Excellent
Lecithin	4.36	0.2150	Excellent
Casein	0.85	1.1058	Excellent
Oxovitelin	0.69	1.3623	Excellent

It is probable that in all of these instances phosphoric acid is first split off from the organic complex by means of some enzyme

secreted by the mold and then utilized in this form Iwanoff<sup>1</sup> has shown that an enzyme can be prepared from this organism which decomposes nucleic acid with the liberation of phosphoric acid and purine bases, and the writer<sup>2</sup> has demonstrated the presence, in another mold, of an erepsin capable of hydrolyzing casein. An enzyme which decomposes phytin into phosphoric acid and inositol is widely distributed in plants, and lecithin is known to be acted upon by an enzyme similar to, if not identical with, lipase. Whether or not such a cleavage must take place before the phosphorus is available can be determined only by further study. The cultural experiments described here show, however, that quite a variety of phosphorus-containing substances may be utilized by *Aspergillus niger* as sources of this element, but that compounds of trivalent phosphorus, though not appreciably toxic, are not available for assimilation by this organism.

<sup>1</sup> *Zeitschr f physiol Chem*, xxiv, p 31

<sup>2</sup> Bureau of Animal Industry, Bulletin 120, p 41

# ON THE APPLICATION OF THE OPTICAL METHOD TO A STUDY OF THE ENZYMATIC DECOMPOSITION OF NUCLEIC ACIDS

BY SAMUEL AMBERG AND WALTER JONES

(From the Laboratory of Physiological Chemistry, Johns Hopkins University )

(Received for publication, June 27, 1911 )

Experiments recently made in this laboratory have shown that nucleic acids undergo enzymatic decomposition in various ways when submitted to the action of the ferments of different gland extracts in fact each gland is somewhat characterized by the manner and extent of the decomposition which it can accomplish. Thus extract of pigs' pancreas was found capable of attacking nucleic acid at two different points, giving rise on the one hand to free purine bases and on the other hand to free phosphoric acid with the consequent formation of two purine nucleosides, one of which remains unaltered (guanine-nucleoside) and is found as an end product, while the other (adenosin-nucleoside) is desamidized<sup>1</sup>. It has also been shown how hypoxanthine may be formed from nucleic acids by the ferments of gland extracts without the intermediate production of adenine<sup>2</sup>. An investigation by W. D. Reddish<sup>3</sup> will show how certain glandular extracts completely decompose nucleic acids with liberation of all their purine groups.

While this work was in progress an article appeared by Pighini<sup>4</sup> who found that when yeast nucleic acid is digested at the body temperature with blood serum the optical rotation of the substance is rapidly diminished, finally falling near or quite to zero and this writer was undoubtedly of the opinion that diminution in rotary

<sup>1</sup>W. Jones. *This Journal*, ix, p. 169.

<sup>2</sup>Amberg and Jones. In publication. *Zeitschr. f. physiol. Chem.*

<sup>3</sup>To be published.

<sup>4</sup>Pighini. *Zeitschr. f. physiol. Chem.*, lxx, p. 85.

power was brought about by a complete disruption of the molecule of nucleic acid

It thus became desirable to know how far the results which we had obtained with gland extracts were to be ascribed to the serum which they contained. Many of our experiments had been made with thymus nucleic acid (some with the sodium salt) and numerous experiments had convinced us that blood serum exercises no effect whatever upon this substance, for after digestion with the sera of a number of different animal species, a large part of the nucleic acid could be recovered and shown to conduct itself polarimetrically and chemically like the initial nucleic acid, while no decomposition products of the substance could be found. Nevertheless, to confirm our former results experiments were made with extracts of organs that had been freed from blood by perfusion but it was uniformly observed that these extracts exerted the same enzymatic action upon nucleic acid that had been shown with organs containing blood<sup>1</sup>. As might have been supposed, enzymatic activities, which are different in different organs, could not possibly be brought about by blood serum which is present in them all.

We next proceeded to study the action of blood serum upon thymus nucleic acid by the optical method which Pighini had employed but could observe no change whatever in the optical rotation of this substance when it was digested with the blood sera of the ox, dog or rabbit. We finally made experiments with rabbit's serum and yeast nucleic acid following exactly the directions given by Pighini, and were able to confirm to a certain degree the findings which he reports. Yeast nucleic acid, in distinction from thymus nucleic acid, does suffer a diminution in rotation by digestion with the blood serum of both horse and rabbit. But this does not involve the liberation of either phosphoric acid or purine bases, in a form capable of precipitation by the reagents commonly employed for the purpose as can be done with the products of the action of glandular extracts.

Very recently Levene and Medigreceanu<sup>2</sup> described an extended study of the action of the plasmata of dog's organs upon nucleic

<sup>1</sup> Amberg and Jones *loc cit*

<sup>2</sup> Levene and Medigreceanu *This Journal*, 15, p. 389

acids and their decomposition products One experiment is given on the action of dogs' serum upon yeast nucleic acid, and one upon thymus nucleic acid Their results are practically in accord with those which we obtained with the sera of the horse and rabbit They found very little depression in the rotation of thymus nucleic acid but considerably more in yeast nucleic acid Levene and Medigreceanu also note that their polarimetric observations were always made after the material had cooled to the room temperature, as changes in temperature can noticeably affect the magnitude of the rotation

In the course of our work we took occasion to examine somewhat closely the factors other than ferments that can influence the optical rotation of yeast nucleic acid This had already been done, with thymus nucleic acid<sup>1</sup> whose specific rotation was found to change considerably with change in dilution and to a very marked extent with changes in acidity This was also found to be the case with yeast nucleic acid But the dilution curves of salts of the two nucleic acids are not the same shape, that of yeast nucleate being more nearly a straight line, and the specific rotation of thymus nucleate is very much less than that of yeast nucleate in any given concentration An examination was also made of the influence of temperature upon yeast nucleate a matter which was referred to by Levene and Medigreceanu *We found that the rotation is markedly lowered as the temperature rises* This alteration of the optical rotation of yeast nucleate brought about by changes of temperature is such that the expression "room temperature" becomes somewhat vague A polarimeter tube filled with yeast nucleate solution was allowed to stand in the room and observations of the rotation were made every 24 hours The variations noted (caused evidently by changes of the room temperature) were great enough to suggest a decomposition of the nucleate

But these alterations in rotation are not permanent A solution kept at 20° for several days will promptly show the rotation corresponding to 40° when it is heated to that temperature, and a solution kept at 40° will show the proper rotation for 20° when it is cooled

Pighini states that his initial readings were made at room tem-

<sup>1</sup>W Jones This *Journal*, v, p 1

perature, the digestions carried on in an Ostwald thermostat at 37° and after cooling again to the room temperature readings were again made. But he continues that the experiment succeeds better when the final reading is made at 37°, a remark which suggests that his results are too high. He attributes the fall in rotation to a decomposition of the nucleic acid by a ferment of the serum. While an influence of the serum on yeast nucleic acid cannot be denied, the failure to liberate either phosphoric acid or purine bases would indicate that if we are here dealing with a decomposition of nucleic acid, it is unlikely that either phosphonuclease or purine nuclease<sup>1</sup> is concerned. It would therefore appear that results obtained by the optical method in the study of the decomposition of nucleic acids, unsupported by corroborative evidence, must be interpreted with great caution and especially where the change in optical activity is small and may have been caused by factors not connected with ferment action.

## EXPERIMENTAL

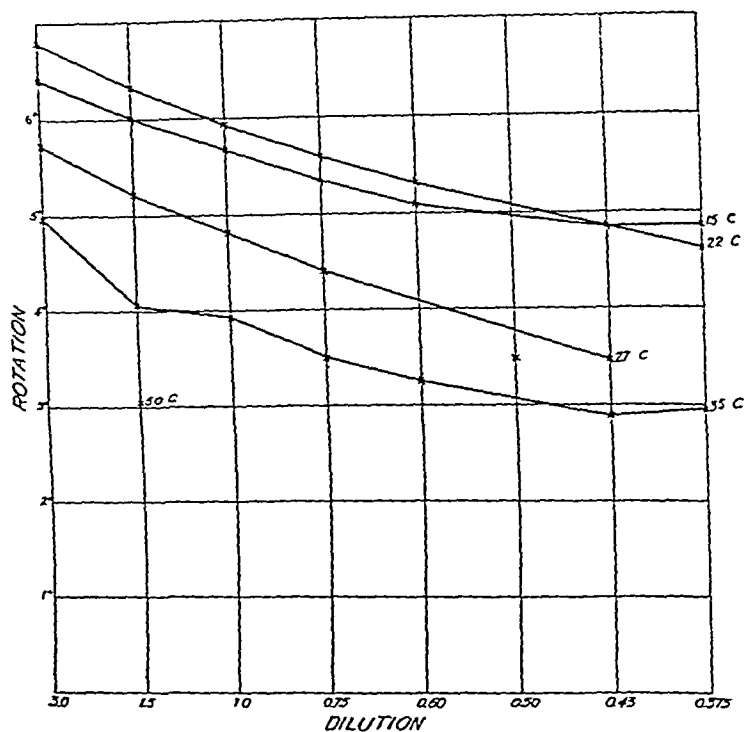
A 3 per cent solution of Merck's yeast nucleic acid was made in water with enough ammonia to neutralize the fluid. Portions of the solution were diluted with 1, 2, 3, 4, 5, 6, and 7 parts of water respectively and the eight solutions examined optically at various temperatures. The results are given in table I and are graphically represented in diagram I after reduction to a common concentration. All observations were made in 2 dm tubes, and all rotations were positive.

TABLE I

CONCENTRATION (PER CENT)	3	1 5	1 00	0 75	0 60	0 50	0 43	0 375
Polarization at 15° C	6 79	3 13	1 97	1 40	1 07		0 68	0 60
Polarization at 22° C	6 42	3	1 88	1 35	1 02		0 70	1 58
Polarization at 27° C	5 71	2 60	1 60	1 10		0 69	0 57	
Polarization at 35° C	4 98	2 07	1 27	0 87	0 65		0 40	0 37

<sup>1</sup>Amberg and Jones *loc cit*

DIAGRAM I



As in the experiment above, a 3 per cent solution of yeast nucleate was diluted with water and for comparison the same solution was diluted with 2.5 per cent ammonia. Results are given in table II, and diagram II.

TABLE II.

*Dilution with water*

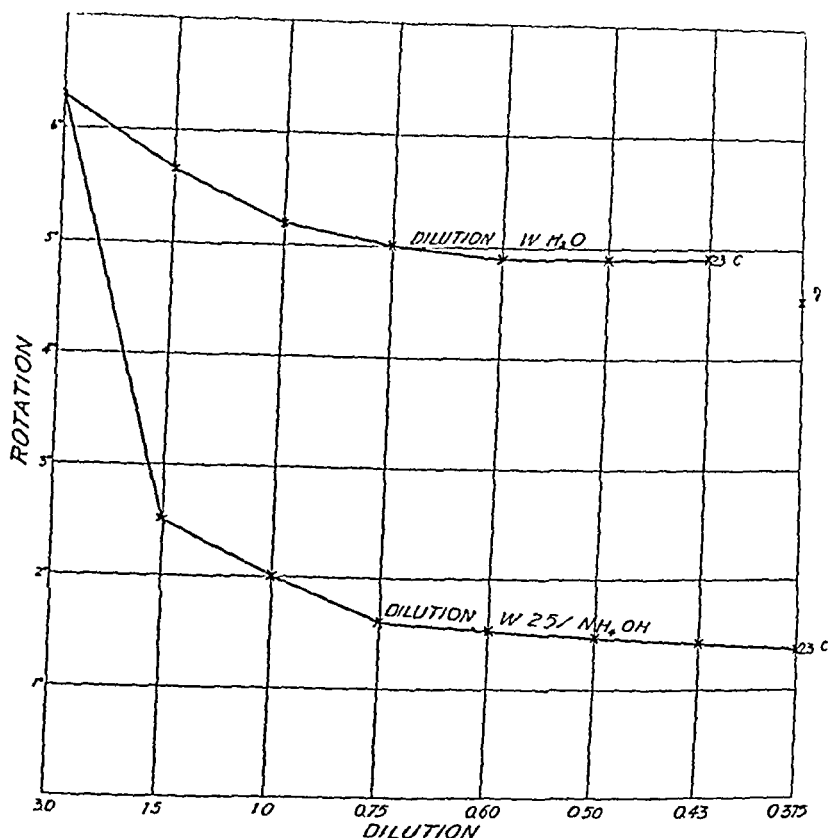
Polarized at 23° C	6.30	2.85	1.78	1.25	0.98	0.80	0.68	0.58
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*Dilution with 2.5 per cent ammonia*

Polarized at 23° C	1.22	0.65	0.42	0.31	0.25	0.21	0.18
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DIAGRAM II



A 16 per cent solution of yeast nucleate was made in normal saline with enough ammonia to neutralize the fluid. Of this 22 cc were treated with 2 cc of rabbit's serum and for a control 22 cc treated with 2 cc of saline.

A solution of thymus nucleate 15 per cent made in normal saline. Of this 22 cc treated with 2 cc of rabbit's serum and for a control 22 cc treated with 2 cc of saline.

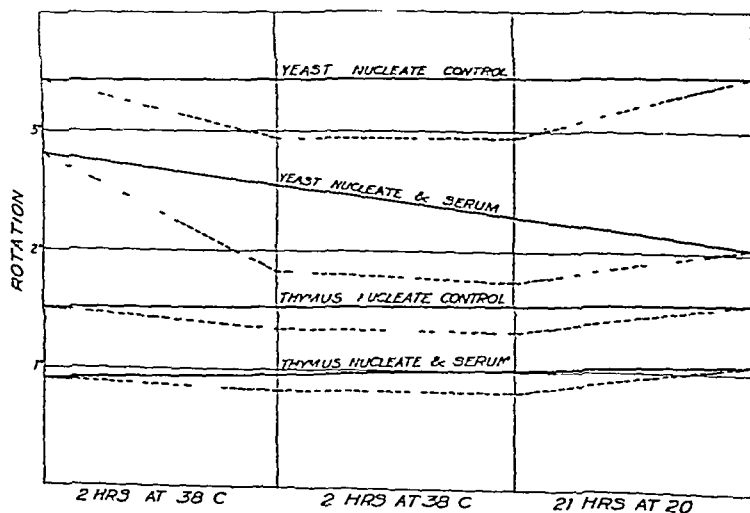
These four solutions were placed in four polarimeter tubes and digested at the body temperature for two periods of two hours each and then allowed to cool in the room for 21 hours. The results are given in table III and diagram III. It will be noted that the control solutions as well as the solution of thymus nucleate suffer a fall in rotation as the temperature rises but after cooling the rotation returns to the initial. On the contrary, the solution of

yeast nucleate and serum does not regain its initial rotation after digestion at 40° and subsequently cooling to 20°

TABLE III

SOLUTION	INITIAL READING	TWO HOURS AT 35°	FOUR HOURS AT 35°	TWO HOURS AT 20°
Yeast nucleate control	3 45	2 93	2 96	3 46
Yeast nucleate and serum	2 80	1 85	1 74	2 08
Thymus nucleate control	1 52	1 35	1 35	1 55
Thymus nucleate and serum	0 95	0 83	0 83	1 08

DIAGRAM III





# STUDIES ON MELANIN II THE PIGMENTATION OF THE ADULT PERIODICAL CICADA (*TIBICEN SEPTENDECIM L*)<sup>1</sup>

BY ROSS AIKEN GORTNER

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(Received for publication, June 30, 1911)

## INTRODUCTION

The Periodical Cicada (*Tibicen septendecim L*), commonly known as the "seventeen year locust," presents one of the most remarkable changes in coloration of which I am aware. The pupæ, at the end of the seventeen-year period of subterranean life, emerge from the ground about dusk on a late spring evening and at once proceed to climb upward upon any object which they may find, such as trees, shrubs, weeds, blades of grass, etc. Here they fasten their claws firmly in the bark or on a leaf and the process of transformation begins. This process can best be described by reference to the accompanying illustrations (Plate 1)<sup>2</sup>. Fig 1 shows the pupa ready for transformation. Fig 2 shows the adult beginning to issue from the pupal shell. Fig 3 shows the adult nearly free from the pupal shell. Fig 4 shows the adult, completely formed but as yet practically colorless. Fig 5 shows the adult with the coloration fully matured several hours after emerging from the pupal shell. The time occupied in the transformation from pupa to adult varies from twenty minutes to an hour or more.

<sup>1</sup>Reported before the Biochemical Section of the American Chemical Society at the Summer Meeting, Indianapolis, Indiana June 28 to July 1, 1911.

<sup>2</sup>This plate is reproduced from Bulletin 71, of the Bureau of Entomology, the U S Department of Agriculture, by the kind permission of C L Marlatt, author of the Bulletin and Assistant Chief of the Bureau.

"The colors of the forming Cicada are a creamy white with the exception of the reddish eyes and two strongly contrasting black patches on the prothorax, a black dash on each of the coxæ and sometimes on the front femora, and an orange tinge at the base of the wings"<sup>1</sup> Gradually this creamy hue turns to a smoky appearance which continues to deepen until in a very few hours the entire insect is a deep black with the exception of the eyes which remain red, and the wing veins which are orange

I had expected from my earlier work on the pigmentation of the meal worm<sup>2</sup> (*Tenebrio molitor*), to find that this change in color was an oxidation, induced by the action of an oxidizing enzyme, and my expectation was fully realized, although there are important differences in the two cases

#### THE MATERIAL

Pupæ and newly emerged adult Cicadas belonging to Brood II (Marlatt, *loc cit*) were collected at Highwood, N J, on the nights of May 29 and 30, and a second portion at Summit, N J, on the night of June 6 Enough newly emerged adults were collected to completely fill a 500 cc bottle containing 100 cc of distilled water The insects were pressed down until they were completely covered by the water and the bottle was then tightly stoppered In this manner all air was excluded

A quantity of the pupæ were also collected and placed in an empty gas wash bottle, carbon dioxide was then passed through until all of the air had been displaced and no signs of life were apparent In this state of "suspended animation" they remained for about eighteen hours, and when they were exposed to the air in this laboratory they nearly all revived and a considerable number transformed in the normal manner By this means I was able to obtain adults which had just emerged and to watch the process of coloration without the aid of artificial light

#### EXPERIMENTAL

The pigmentation of the Cicada is an oxidation, and the color is due to the action of a tyrosinase acting on some aromatic amino

<sup>1</sup> Quoted from Riley, cited by Marlatt, *The Periodical Cicada*, *loc cit*

<sup>2</sup> Gortner *This Journal*, vii, p 365, 1910

phenol<sup>1</sup> producing, as a result of the reaction, a black, insoluble pigment. That the reaction is an oxidation can be shown in several ways.

1 If the insect which has just emerged from the pupal shell is submerged in water, no coloration appears, and the Cicada remains colorless until decomposition sets in. If, however, the submerged insect is removed from the water and exposed to the action of the air before decay has set in, the coloration proceeds at once.

2 If the colorless adults are kept in a stream of carbon dioxide no coloration is observed, but when the carbon dioxide is displaced by pure oxygen, blackening readily appears.

In the meal worm I found that the tyrosinase was present in the body filling, but I have been unable to find a tyrosinase in the body filling of either the pupa or the adult Cicada. The body filling, when exposed to the air, does not darken nor does the addition of tyrosine cause a coloration, the addition of guaiacol however, causes a deep red coloration while no coloration appeared in a boiled check. This would show that there is apparently some sort of oxidase present there.

The method by which the tyrosinase was isolated is as follows. The water in which the Cicadas had been collected was drained off after about twelve hours and was found to be a very powerful solution of an oxidase and a chromogen, for when it was exposed to the action of the air it rapidly turned jet black, without producing any intermediate pink coloration. The addition of three volumes of alcohol throws down a dense grey precipitate which has no oxidizing properties, or rather a precipitate whose oxidizing properties have been destroyed by the alcohol. Saturation with ammonium sulfate, however, gives a grey precipitate which is readily soluble in water to a clear solution, and which rapidly oxi-

<sup>1</sup> I have stated above that the chromogen is an "aromatic amino phenol." This statement can be substantiated by the fact that, whereas tyrosine gives first a red and then a black in the presence of tyrosinase, tyrosol, prepared from tyrosine by the action of yeast, which splits out CO<sub>2</sub> from the carboxyl and replaces the amino group with hydroxyl, gives only a pink to red in the presence of tyrosinase, showing that the primary red coloration of tyrosine is probably due to the phenolic hydroxyl, and the final black to some reaction of the amino group.

dizes tyrosine to a jet black precipitate, produces a rose coloration with tyrosol, pink with guaiacol, and deep blue with tincture of gum quaiac. Boiled checks were carried out in all cases and in no instance was coloration observed.

When a portion of the solution was heated at 75° for one minute all oxidase activity was destroyed and no coloration was produced on the subsequent addition of tyrosine, heating at 50° and 60° for a similar period did not appear to affect the activity, while one minute's exposure at 70° decidedly weakened the oxidizing power. The same results were found when the colorless adults were used, heating for one minute at 75° totally inhibits the coloration. All oxidase activity is also lost if the tyrosinase solution is dialyzed in collodion bags in a stream of running water for twelve hours. This is in accord with Porter's observations<sup>1</sup> on other enzymes, such as pepsin, trypsin, rennet, emulsion, etc., and he suggests that the ferment is changed, by contact with the membrane, into an anti-ferment, which inhibits the reaction.

*The Effect of Light on the Pigmentation.* Several colorless adults were exposed to strong light, dim light, total darkness and light which had passed through blue glass. No apparent difference was noted in the rapidity of coloration nor in the final depth of color.

*The Origin of the Tyrosinase.* In the meal worm I found that the normal coloration developed after the death of the larva, a condition which was probably due to the fact that the oxidase and chromogen are both to be found in the body filling in sufficient quantity to produce the normal coloration even after the secretion of the enzyme has ceased. In the Cicada the enzyme, once formed, does not depend upon life processes, but life is necessary to produce the normal coloration. This is readily accounted for by the supposition that the oxidase is secreted together with the new cuticula, and in the absence of life processes the secretion of the cuticula ceases. Colorless adults which were thoroughly washed and rubbed in a stream of water, after death, in order to remove as completely as possible the newly formed cuticula, darkened only in isolated spots, mostly in the folds of the abdomen where the cuticula had probably not been completely removed, while other

<sup>1</sup>Porter *Biochem Zeitschr*, xxv, p 301, *Quart Journ of Exp Physiol*,  
iii p 375

adults which had been as thoroughly washed, but not killed, slowly darkened to the normal color. The assumption that the oxidase is formed at the same time that the cuticula is secreted would explain why no tyrosinase was obtained from the body filling of the pupæ, or adults.

*The Chromogen* I have been unable to identify the chromogen which is oxidized by the tyrosinase. Perhaps it is tyrosine or some similar body such as the unknown component of the keratin molecule<sup>1</sup>. When the water in which the adults were collected was precipitated by alcohol, filtered, and the filtrate evaporated, a yellowish residue was obtained which gave a strong Millon's reaction and which colored a dusky black on the addition of tyrosinase. The coloration was not as intense, however, as might have been expected.

Another possibility, inasmuch as the coloration is a uniform black over the entire body, is that the entire new cuticula is formed by the reaction between the oxidase and the chromogen, in the same manner that the Japanese lacquer is formed by the action of laccase on the milky latex of the tree *Rhus vernicifera*.<sup>2</sup>

#### SUMMARY

The pigmentation of the periodical Cicada (*Tibicen septendecim* L.), is due to the interaction of a chromogen and an oxidase of the tyrosinase group. The oxidase is not present, as such, in the body of the pupa or adult but is apparently secreted together with the new cuticula, inasmuch as the oxidase is readily isolated from the water in which the newly emerged adults have been washed. The enzyme produces coloration after death, but these colorations do not deepen to the normal color, probably because the secretion of the new cuticula, and as a result the secretion of the enzyme, ceases in the absence of life processes. The tyrosinase is rendered inactive by alcohol and by prolonged dialysis in collodion bags.

<sup>1</sup> Gortner *This Journal*, 11, p. 355, 1911.

<sup>2</sup> Yoshida *Journ Chem Soc*, 1911, p. 472, 1883, Bertrand, *Compt rend acad sci* 1891, p. 1215, 1894.



I wish to thank Doctor J M Nelson, of the Laboratory of Organic Chemistry, at Columbia University, for generously putting all facilities at my disposal so that a part of these observations could be made the same night the material was secured I also wish to thank Mr C W Otto for assistance in collecting a portion of the material



## THE TRANSFORMATION OF THE EMERGED PERIODICAL CICADA

1—Pupa ready for transformation 2—Adult beginning to issue from pupal shell 3—Adult nearly free from pupal shell 4—Freshly transformed adult the coloring immature 5—Adult, several hours after transformation, the coloring mature About natural size



# FURTHER EXPERIMENTS UPON THE DETERMINATION OF IODINE IN THYROID

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(Received for publication, June 18, 1911 )

It was pointed out some years ago by F C Koch,<sup>1</sup> that the iodine content of desiccated sheep thyroids prepared at different seasons of the year was as much as three times as great in the winter as in the summer months. It appeared therefore of interest to ascertain if a similar seasonal variation could be observed in the iodine content of the thyroid of some other animal. For this purpose there were obtained samples of thyroids at intervals of two weeks, from the dogs killed at the Washington dog pound during the year 1910 and part of 1911. The samples consisted of from 10 to 32 single glands in each case, they were dried, powdered, and after all had been obtained, simultaneously analyzed by the new Hunter iodometric method.<sup>2</sup> In connection with this experiment an extensive comparative investigation of the Hunter and Baumann methods for the determination of iodine in thyroid was made, as well as additional determinations by the modification of the Baumann method suggested by Riggs.<sup>3</sup>

The present work demonstrates the superiority of the Hunter method, both as to reliability of results and simplicity of manipulation, and therefore diminishes the importance of the question raised by Riggs in regard to the possibility of the occasional production of iodate during the fusion and the consequent loss of iodine by the Baumann method. A few remarks in this connec-

<sup>1</sup> Koch *Proc Amer Pharm Assoc*, 17, p 371, 1907

<sup>2</sup> Hunter *This Journal*, 11, pp 321-349, 1910

<sup>3</sup> Riggs *Journ Amer Chem Soc*, 31, p 710, 1909, 32, p 692, 1910

tion may not be out of place at this time, since the admission of Riggs' hypothesis would discredit the work of all investigators who have hitherto used the Baumann method furthermore, in his second paper Riggs,<sup>1</sup> has misunderstood certain details of my previous experiments and therefore assumes that they do not disprove his contention

For instance he states that I claimed complete extraction of the acidified aqueous solution with three 2-3 cc portions of carbon tetrachloride, and furthermore that I have adopted such amounts of carbon tetrachloride instead of the usual 10 cc quantity employed by everyone else The title and headings of my table,<sup>2</sup> nevertheless show very clearly that 2-3 cc quantities of this solvent were used only "to remove residual I from aqueous layer," that is, these small quantities of  $\text{CCl}_4$  were used upon those solutions which were left from the regular iodine determination, and which had therefore already been shaken out with the usual 10 cc portions of  $\text{CCl}_4$  These smaller amounts were used only to furnish a more delicate indication of the quantity of iodine present, since more color is imparted to a smaller volume of  $\text{CCl}_4$  by a given amount of iodine In this way it was hoped to be certain of as complete removal of iodine as possible before the application of the reduction process with the Devarda's alloy It will be noticed however that additional minute amounts of iodine were found (last column of table), after making alkaline and again acidifying, both in the cases where the alloy had been used and in those in which none was added Thus showing that even where the greatest care was exercised to remove all free iodine, an additional amount could be recovered after simply increasing the concentration of the salts in the aqueous solution without the aid of any reduction whatever Riggs appears never to have made such obvious controls I believe these quantities are of just about the order of magnitude measured in most cases by Riggs, and attributed by him to iodine recovered from iodate This appears more probable since in addition to using fresh glands, which no doubt frequently corresponded to smaller amounts of dried thyroid than generally used for analysis, he extracts the iodine from aliquot portions representing only one-tenth of his sample, thus necessitating the estimation of considerably less iodine than most investigators attempt To do this he has adopted colorimetric standards of about one-tenth the usual strength, but with such small amounts of iodine the difficulty is not so much in the colorimetric estimation as in the complete extraction by the immiscible solvent These attempted refinements which he has adopted are probably the cause of the most of the difficulties he has so far experienced with the Baumann method The figures he gives in his tables are in terms of milligrams iodine per gram of fresh gland, or in the sample used for

<sup>1</sup>Riggs *loc cit*

<sup>2</sup>*Journ Amer Chem Soc*, 1911, p 1328

analysis, but since the weights of the gland or sample are not given for any analysis, the actual quantities of iodine upon which he bases his conclusion in regard to the need of the reduction process can not be calculated

There is no direct way to prove the presence or absence of iodate in the melt obtained by the Baumann fusion of thyroid, since too many interfering substances are present. There is, however, some apparently very good evidence against its production. Thus, during the combustion of the organic material of the sample a large quantity of oxygen is necessarily required that all of this does not come from the air is shown by the active reduction of much of the nitrate to nitrite. Now, if the nitrate is so actively reduced, why should not the iodate, if present at all, be simultaneously reduced to iodide? In fact a number of experiments quoted by me,<sup>1</sup> demonstrate that iodate itself yields all its iodine when mixed with organic material and analyzed by the Baumann method. There are a number of circumstances other than the doubtful production of iodate which might possibly explain the irregular results obtained by Riggs. The one I suggested in my previous paper still appears to me to be the most plausible, even in spite of Riggs' later communication which is hardly more than an additional amount of exactly the same kind of evidence as that already presented. Perhaps the real difficulty is after all the result of his efforts to increase the delicacy of the Baumann method beyond its limitations, this appears most probable since in so far as it is possible to ascertain from his results, his greatest variations are obtained with the smallest actual quantities of iodine.

Riggs and Beebe state that they have, by the use of the reduction process, frequently found iodine in glands which they would otherwise have considered "iodine-free," and they question the conclusions drawn from experiments with "iodine-free" glands. I have examined a great number and variety of thyroids and have never failed to find iodine except in two samples of thyroid, one from children, and the other from the aoudad, glands upon which Riggs makes no report. The frequent failure of these authors to find iodine by the ordinary method suggests that they have not used the Baumann method properly, as witness for instance their

<sup>1</sup>Seidell *Journ Amer Chem Soc*, cxi, p 1323

adoption of considerably weaker colorimetric standards and choice of aliquot portions, representing quantities of thyroid from which it would not be expected that appreciable amounts of iodine could be obtained. As Hunt and I explicitly stated<sup>1</sup> we used the term "iodine-free" only in the sense in which Baumann and many other writers have used it, and from which many drew the conclusion (which we were opposing) that iodine is an unimportant or accidental constituent of the gland, that is, as we stated, we called the thyroid "iodine-free" when one or two grams tested by the Baumann method failed to give even a qualitative test for iodine. We stated that by using larger amounts of material and special methods, iodine can be found in practically all organic matter, and recognized that the question whether the activity of the "iodine-free" thyroid was due to the traces of iodine which could be found by these special methods could not be definitely answered. Since, however, thyroid of this character had only about the activity of certain other organs of internal secretion, the prostate for example, (with the activity of which iodine has not been thought to be associated), and since the activity of thyroid increased greatly and progressively with its iodine content, we thought it probable that this thyroid had a low degree of activity aside from the traces of iodine present. This question, which can evidently not be definitely answered, is scarcely worth the emphasis which has been placed upon it, it is evident that the conclusions drawn by a number of writers that iodine is not an important constituent of the gland (and this was the question of most interest to us), is not tenable whichever standpoint is held.

It was perhaps an oversight on my part not to explain in my last paper exactly how my fusions are made. Descriptions of the principle of the Baumann method have been given so often, however, that I considered it unnecessary, especially since I specified the most essential point which must be observed, viz, the obtaining of a clear tranquil melt, and furthermore none of my results give evidence of irregularities due to iodate. To obtain a clear melt, I use a fusion mixture composed of 5 parts by weight of sodium hydroxide to 1 part of potassium nitrate, both finely powdered and thoroughly mixed. Of this mixture there are used 6 to 7 grams per 1 gram of sample, although a considerably larger proportion is not objectionable, after stirring the dry materials together, enough distilled water to yield a

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<sup>1</sup> Bulletin 47, Hygienic Laboratory, U S Public Health and Marine Hospital Service, 1907

rather thin paste is added and the stirring continued. The fusion is conducted very slowly and a few pinches of potassium nitrate are usually thrown in at the end to destroy any particles of unburned carbon. Nickel crucibles were used in all cases.

In the paper by Hunter, describing his new volumetric method, there are given comparative results upon two samples of sheep thyroid in which the iodine had been previously determined by me by the Baumann process. These results showed satisfactory agreement in one case and not in the other. I have since made redeterminations on these two samples and practically all others on hand at our laboratory. The results of Hunter were confirmed. In regard to the Hunter method, the very admirable description of its various details was followed by me in practically every particular, in fact, after a very extensive experience with it, I am unable to offer a single essential improvement.

The determinations by the Baumann method were made as follows:

The melt obtained by the fusion as above described was dissolved in about 100 cc of  $H_2O$ , and the solution filtered into a separatory funnel, acidified with sulphuric acid (1 volume of  $H_2SO_4$  d 1.84 to 1 volume  $H_2O$ ), which was added very gradually until a piece of litmus paper floating in the liquid just turned red. An additional 10 cc of the sulphuric acid added, 10 cc of  $CCl_4$  then introduced and the mixture carefully shaken, 10 drops of 1 per cent  $NaNO_2$  solution then added and the shaking continued at intervals for several minutes. Enough of the  $CCl_4$  layer was filtered into a cup of a Duboscq colorimeter to about half fill it, and the intensity of the pink color compared with that of a standardized aqueous Fuchsine S solution prepared as already described by me,<sup>1</sup> to correspond in intensity of pink color to that of 10 mg iodine dissolved in 10 cc  $CCl_4$ .<sup>2</sup> About 10 readings were usually made and the average of them all taken. If the sample contained much more than 0.1 per cent of iodine, either a smaller quantity than 10 gram was used for the determination, or a larger quantity than 10 cc of  $CCl_4$  was used to extract the liberated iodine.

In table I, are given the comparative results obtained upon commercial desiccated thyroids and samples prepared in the laboratory from thyroids obtained from different sources. Samples

<sup>1</sup>Seidell *This Journal*, III, p 391, 1907

<sup>2</sup>It should be mentioned in this connection that a restandardization of my Fuchsine S solution showed that it had not changed appreciably in intensity or shade of pink color during more than one and one-half years.



TABLE I

*Comparative Results of the Determination of Iodine in Desiccated Thyroids,  
by the Hunter and Baumann Methods*

SAMPLE NO	THYROID OF	PER CENT IODINE BY METHOD OF	
		Baumann	Hunter
99	Sheep (P D & Co )	0 179	0 185
99(1)	Sheep (P D & Co )	0 176	0 185
100	Sheep (P D & Co )	0 170	0 188
101	Sheep (P D & Co )	{ 0 063 } (0 103)	0 153
102	Sheep (P D & Co )	0 125	0 162
103	Sheep (P D & Co )	0 192	0 219
104	Sheep (Armour & Co )	0 106	0 138
105	Sheep (P D & Co )	0 110	0 120
106	Sheep (P D & Co )	0 196	0 218
106(b)	Sheep (P D & Co )	0 210	0 212
107	Sheep (Armour & Co )	0 130	0 145
108	Sheep (Armour & Co )	0 125	0 134
109	Sheep (Armour & Co )	0 130	0 141
109(a)	Sheep (Armour & Co )	0 110	0 142
114	Beef (Armour & Co )	0 260	0 283
115	Hog (Armour & Co )	0 330	0 422
116	Sheep (P D & Co )	0 11	0 118
117	Sheep (P D & Co )	0 11	0 117
118	Sheep (P D & Co )	0 12	0 158
119	Sheep (Armour & Co )	0 11	0 135
120	Sheep (Armour & Co )	0 10	0 129
121	Sheep (Armour & Co )	0 11	0 140
130(a)	Sheep (Armour & Co )	0 043	0 056
130(b)	Sheep (Armour & Co )	0 046	0 048
131(b)	Sheep (B W & Co )	0 099	0 112
132	Sheep (P D & Co )	0 054	0 062
132(a)	Sheep (P D & Co )	0 027	0 032
143(2nd)	"Thyraden" (Knoll)	0 085	0 100
345	Sheep (Armour & Co )	0 249	0 279
346	Sheep (Armour & Co )	0 083	0 095
347	Sheep (Armour & Co )	0 192	0 212
348	Sheep (Armour & Co )	0 140	0 162
349	Sheep (Armour & Co )	0 155	0 146
350	Sheep (Armour & Co )	0 244	0 271
351	Sheep (Armour & Co )	0 186	0 202
352	Sheep (Armour & Co )	0 209	0 231
353	Sheep (Armour & Co )	0 197	0 215

Samples No 130(a) to 132(a) are commercial thyroid tablets

TABLE I Continued

SAMPLE NO	THYROID OF	PER CENT IODINE BY METHOD OF	
		Baumann	Hunter
354	Sheep (Armour & Co )	0 147	0 144
355	Sheep (Armour & Co )	0 252	0 252
356	Sheep (Armour & Co )	0 198	0 219
Ser I, '07	Dog	{ 0 13 (0 176) }	0 218
Ser IV, '07	Dog	0 15	0 175
Ser V, '07	Dog	0 30	0 358
Ser 6, '08	Dog	0 21	0 277
Ser 7, '08	Dog	0 10	0 131
Ser 8, '08	Dog	0 12	0 166
23807	Human	0 26	0 329
23785	Human	0 45	0 588
23821	Human	{ 0 23 (0 384) }	0 430
23927	Human	0 05	0 051

99 and 121 are the two of which portions were analyzed by Hunter. The determinations by the Baumann method were, with the exception of those for samples 345-356, made two years or more ago. In some cases the older determinations gave results unaccountably low, but in practically every one of these cases subsequent determinations (given in parentheses), show higher values more nearly in accord with the results by the Hunter method. It will be noted that the Baumann method gives results which vary from about 80 to 95 per cent of those obtained by the Hunter method, about two-fifths being approximately 80 per cent and three-fifths 90 per cent or more, of the latter results. This is a somewhat greater variation than permissible in check determinations upon the same sample, and indicates that the Baumann method does really give low results.

In order to obtain more information upon this point, and at the same time to test further the hypothesis of Riggs that the low results are due to production of iodate from which the iodine can be again recovered by the reduction process with the aid of Devarda's alloy, the following experiment was made. It should first be mentioned, however, that it appeared unnecessary to make

further experiments upon the recovery of iodine from the acid aqueous solution remaining from the Baumann determination, since a number of recent attempts to recover iodine from them confirmed entirely the negative results already published by me<sup>1</sup> It was therefore decided to modify the procedure by introducing the reduction step (with Devarda's alloy) before the extraction with  $\text{CCl}_4$ , and compare the results with those obtained upon aliquot portions of the same solutions not previously reduced with Devarda's alloy

Those samples shown in table I, which gave the greatest variations by the two methods, were used for the determinations In each case the fusion was made by the Baumann method, using however, somewhat larger amounts of sample and fusion mixture than usual, viz, 15 to 20 grams of sample and 12 to 20 grams of the fusion mixture composed of five parts  $\text{NaOH}$  and one part  $\text{KNO}_3$  The melts were dissolved in water and the solution diluted to 200 cc in every case Of these solutions, 50 cc portions were withdrawn for the regular Hunter method, 75 cc for the Baumann colorimetric determination as described above, and the remaining 75 cc treated with 0.5 to 1.0 gram of Devarda's alloy and allowed to stand 24 hours, after which time the iodine was determined by following exactly the same details of the Baumann colorimetric method as used upon the 75 cc of solution not previously reduced with the Devarda's alloy The results are given in table II For the sake of comparison the previous results on these same samples shown in table I, are also given in parentheses

In this table it will be seen that the Baumann method still gives results below those obtained by the Hunter method, and since the solutions were the same in both cases it must be assumed that the low results by the Baumann method are due to incomplete recovery of the iodine which had been converted to the water-soluble condition That the lower results can not be due to iodate is shown by the similarly low results obtained in the solutions which had been subjected to the reduction process with Devarda's alloy Attention should be called to the very close agreement between the duplicate determinations made by the Hunter method in contradistinction to the variation in the two sets of results by

<sup>1</sup>*Journ Amer Chem Soc*, xxxi, p 1326, 1909

TABLE II

*Comparison of the Hunter, Baumann and Baumann Reduction (Devarda's Alloy) Methods on Aliquot Portions of the Aqueous Solution of the Melt obtained by Fusion of the Sample of Thyroid with the Baumann Fusion Mixture (5 NaOH 1 KNO<sub>3</sub>)*

SAMPLE NO	WEIGHT TAKEN	CHAMBERLAIN FUSION MIXTURE	PER CENT IODINE FOUND BY		
			Hunter volumetric method	Baumann colorimetric method	Baumann (with reduction) colorimetric method
Dried Beef and 0.1 per cent I	2.0	14.0	0.094	0.085	not det
Dog thyroid no 23	1.0	6.0	0.242	0.250	not det
Dog thyroid no 23	1.0	12.0	0.263	0.260	not det
Dog thyroid no 23	1.0	18.0	0.278	0.244	not det
Sheep thyroids no 101	1.5	12.0	0.155 (0.153)*	0.103	0.105
Sheep thyroids no 102	2.0	20.0	0.164 (0.162)	0.124 (0.125)	0.110 (slight loss)
Sheep thyroids no 109 (a)	1.5	12.0	0.139 (0.142)	0.139 (0.110)	0.129
Sheep thyroids no 118	1.5	12.0	0.157 (0.158)	0.154 (0.12)	0.141
Sheep thyroids no 119	2.0	20.0	0.139 (0.135)	0.105 (0.11)	0.113
Sheep thyroids no 120	2.0	20.0	0.132 (0.129)	0.108 (0.10)	0.099
Sheep thyroids no 121	1.5	12.0	0.145 (0.140)	0.128 (0.11)	0.122
Dog thyroids 1, '07	1.0	10.0	0.209 (0.218)	0.176 (0.13)	0.166
Dog thyroids VII, '08	2.0	20.0	0.131 (0.131)	0.093 (0.10)	0.102
Dog thyroids 8, '08	1.5	12.0	0.171 (0.166)	0.157 (0.12)	0.152
Human thyroids 23785	0.5	10.0	0.581 (0.588)	0.532 (0.45)	0.551
Human thyroids 23807	1.0	10.0	0.370 (0.329)	0.296 (0.26)	0.299
Human thyroids 23821	0.5	10.0	0.496 (0.430)	0.384 (0.23)	0.387

Results in parentheses taken from Table I.

the Baumann method. In the case of the latter, however, it must be remembered that the first set of the duplicate determinations were made two and in some cases four years ago during my earlier experience with the Baumann method, also that in the later determinations larger absolute amounts of fusion mixture were used and aliquot portions instead of the whole of the aqueous solutions of the melt were employed.

A few determinations of which those upon the dog thyroid no 23, shown in table II, are examples, seem to show that with both

the Hunter and Baumann methods an increase in the relative proportion of fusion mixture gives higher values for iodine content. The experience gained so far appears to indicate that the proportion of fusion mixture suggested by Hunter, viz., 20 grams per gram of sample, is the most satisfactory amount to use.

Attempts to ascertain to what particular detail of the Baumann method the low results could be ascribed, were unsuccessful. The occurrence of low results is so irregular that all experiments involving the control of particular steps of the method have failed to locate the source of the trouble. As a general thing, however, it may be said that plenty of fusion mixture should be used, but care taken not to have too great an excess of nitrate present, otherwise the large amount of nitrite which will be produced, causes the liberation of sufficient nitrous acid to modify the tint of the  $\text{CCl}_4$  solution of the iodine. Apparently the most critical step of the process from the standpoint of loss of iodine is the acidification of the solution before shaking out with  $\text{CCl}_4$ . The  $\text{CO}_2$  which is evolved must certainly carry along a portion of the free iodine which is liberated at the same time. In fact, Hunter experienced this difficulty,<sup>1</sup> and it was only after adopting the plan of providing an excess of chlorine (which also removed the nitrite), at the time of acidification, that he was able to obtain satisfactorily agreeing results. The success of Hunter's method is undoubtedly due more to this point than to any other improvement he has made. If a similar device could be applied to the Baumann method, the greatest source of error would probably be eliminated.

#### THE IODINE CONTENT OF DOG THYROIDS

As mentioned in the opening paragraph of this paper, an experiment was planned with the object of ascertaining if there is a seasonal variation in the iodine content of dogs' thyroid. Although the results of the experiment have not been conclusive on account of the relatively small number of dogs' thyroids available, a brief description of the work will be given.

In table III are presented the details of the results upon the first series of samples of thyroids which were obtained. About one-half of the dogs were fox terriers, while curs, bulls, pugs,

<sup>1</sup>Hunter *loc cit*, p 330

TABLE III

*Showing the Iodine Content of Samples of Thyroid obtained from Dogs killed at the Washington Dog Pound Series 1*

NO OF SAMPLE	DATE OBTAINED 1910		NO OF SINGLE GLANDS	WEIGHT IN GRAMS		PER CENT DRY WEIGHT	PER CENT IODINE IN DRY SAMPLE
				Fresh	Dry		
1	Jan	17	12	7 444	1 867	25 1	0 150
2	Feb	2	10	7 155	1 654	23 1	0 078
3	Mar	3	22	20 772	4 943	23 8	0 119
4	Mar	14	26	23 650	6 058	25 6	0 139
5	Mar	30	18	18 25	4 952	27 1	0 202
6	Apr	15	18	11 402	3 231	28 3	0 260
7	May	2	32	30 724	8 123	26 4	0 184
8	June	2	20	19 422	4 738	24 4	0 206
9	June	16	16	17 289	4 183	24 2	0 202
10	July	1	20	17 764	4 309	24 2	0 125
11	July	15	18	26 278	6 391	24 3	0 158
12	Aug	2	12	16 300	4 361	26 7	0 154
13	Aug	16	16	10 192	2 621	25 7	0 202
14	Sept	1	18	14 507	3 249	22 4	0 103
15	Sept	16	12	13 389	2 988	22 3	0 093
16	Oct	1	16	12 565	3 119	24 8	0 198
17	Oct	17	12	6 720	1 514	22 6	0 117
18	Nov	3	12	12 540	2 948	23 5	0 077
19	Nov	18	20	17 244	4 223	24 5	0 108
20	Dec	1	12	11 482	2 804	24 4	0 181
21	Dec	17	14	8 777	2 178	24 8	0 104
22	Jan 3, 1911		16	12 838	3 274	25 5	0 117

poodles, hounds, and mixed breeds, formed the remainder. The dogs were killed at the pound in a carbon monoxide fume box and the thyroids removed immediately thereafter. The glands composing a given sample were in every case first cleaned by cutting away adhering tissue, etc., and then weighed as promptly as possible on counterpoised weighing glasses. They were then chopped fine with scissors and placed in a vacuum desiccator containing concentrated  $\text{H}_2\text{SO}_4$ . The drying was practically complete in two to three days, after which time, the residues were weighed and the percentage of dry weight calculated. The material was then ground in a mortar, sifted through a no. 60 sieve, and thoroughly mixed before being placed in the sample bottle. When all of the samples had been obtained, they were placed in a

single large vacuum desiccator and dried to constant weight as determined by weighing from time to time, one of the samples contained in a weighing bottle. This loss during final drying amounted to about 5 per cent, but is not to be deducted from the dry weight of the sample shown in the table, because part of this moisture was no doubt absorbed during the grinding and sifting of the samples. The determination of the iodine in the twenty-two samples was made by the Hunter method, using 1 gram of sample and 20 grams of fusion mixture in every case. The per cents of iodine found show the greatest possible irregularity, and even when plotted on cross-section paper give no indication of a consistent seasonal variation. They indicate that the individual variations of percentage of iodine in the thyroid of dogs must be exceedingly great, and it therefore appeared of interest to analyze individually the thyroids taken from a number of dogs. A second series of samples were therefore obtained, prepared and analyzed as in the case of the first series. The results are given in table IV.

TABLE IV

*Showing the Iodine Content of Thyroids obtained from Dogs killed at the Washington Dog Pound Series 2*

NO OF SAMPLE	DATE OBTAINED 1911	NO OF SINGLE GLANDS	WEIGHT IN GRAMS		PER CENT DRY WEIGHT	PER CENT IODINE IN DRY SAMPLE
			Fresh	Dry		
23	Jan 28	18	27 83 (low)	8 145	29 4	0 275
24	Jan 30	2	2 02	0 446	22 0	0 096
25	Jan 30	2	2 50	0 533	21 3	0 058
26	Jan 30	2	1 325	0 247	18 7	0 038
27	Jan 30	2	1 04	0 25	24 0	0 202
28	Jan 30	2	0 57	0 129	22 6	0 166
29	Jan 30	2	3 82	0 841	22 0	0 036
30	Jan 30	2	0 93	0 225	24 2	0 271
31	Feb 1	20	17 910	4 460	24 9	0 132
32	Feb 3	14	19 28	4 435	23 0	0 092
33	Feb 10	20	19 57	4 819	24 6	0 182
34	Mar 3	20	12 46	3 448	27 6	0 246

As was expected, this series showed even wider variations than the preceding one, which represents averages of larger numbers of thyroids. On the other hand, the individual variations were

found to be considerably greater than anticipated. Thus, of the seven dogs of which the thyroids were analyzed separately (nos 24-30), all were terriers or curs and with one exception (no 25), weighed between about 15 and 20 pounds each, nevertheless the percentage of iodine varied from 0.036 to 0.271. Of the causes which may account for this wide variation among individual dogs, the diet is probably of greatest importance, then follow such factors as age, breed, health, habits, etc.

It may be concluded that if there is a seasonal variation in the iodine content of the thyroids of dogs, the individual variations under ordinary conditions are far too great to permit its detection by analyses of the collected glands from a reasonable number of dogs, moreover, the possibility of sufficiently controlling the conditions to be able to detect a seasonal variation, appears doubtful.

#### THE IODINE CONTENT OF SHEEP THYROIDS

In regard to the observation upon sheep made by Koch<sup>1</sup> it is noteworthy that he has so far never published the data upon which his observation was based. It is of course quite probable that the individual variation with sheep such as reach the large slaughtering houses, is considerably less than with dogs, and furthermore the available number of glands upon which his averages were based are sufficiently great to diminish the individual variations to a minimum. It is entirely possible therefore that Koch's observation is well founded and not the result of an accidental circumstance. Confirmatory evidence will certainly be necessary, however, before the point can be considered as established. In this connection some recently published analyses by Simpson and Hunter<sup>2</sup> should be mentioned. These authors found the fresh weights of the thyroids of 10 sheep to vary from 1.6 to 24.7 grams, and the percentage of iodine in the dried samples from 0.048 to 0.383, thus a somewhat wider variation than found by me for the seven dogs reported in table IV.

Recently we obtained through the kindness of the firms of Armour and Company, and Parke, Davis and Company, representative samples of the several lots of their desiccated Thyroid

<sup>1</sup> *Loc cit*

<sup>2</sup> Simpson and Hunter *Quart Journ Exp Physiol*, iii, p 126, 1910



U S P, prepared by them at various dates during the past year and a half. These samples were sent particularly for use in the work upon the standards for the next edition of the U S P, but in reply to our special inquiry concerning the dates, source of the material, and details of the preparation of the glands, we have been enabled to present additional data upon the question of the seasonal variation in the iodine content of sheep thyroids. The results are given in the following table.

TABLE V

*Showing the Iodine Content of Desiccated Sheep Thyroids Prepared by two American Firms at Various Dates of the Years 1909 to 1911*

LABORATORY NO OF SAMPLE	DATE STAMPED ON BOTTLE	PER CENT IODINE FOUND	SOURCE
345	Dec 16, 1909	0.279	Armour and Company
346	Jan 23, 1910	0.095	
347	Feb 15, 1910	0.212	
348	Apr , 1910	0.162	
349	May, 1910	0.146	
350	June, 1910	0.271	
351	July, 1910	0.202	
352	Aug , 1910	0.231	
353	Sept , 1910	0.215	
354	Oct , 1910	0.144	
355	Nov , 1910	0.252	Parke, Davis and Com pany
356	Jan 16, 1911	0.219	
358	Winter 1910-11*	0.206	
359	Winter 1910-11*	0.206	
360	Winter 1910-11*	0.154	
361	Winter 1910-11*	0.214	

\*From statement in letter of May 4 1911 to the effect that the samples represent lots prepared during the past few months.

According to the information furnished, the thyroid samples (table V), represent selected glands from which abnormally large or small ones were removed. The uncertain part about them is that the glands from both sheep and lambs were used, and this one fact is enough to explain the very marked seasonal irregularity shown in the table. Although these results do not disprove Koch's statement, they indicate that unless he exercised particular care to have only the glands from sheep in his average lots, his results must have been due to an accidental regularity.

# THE EREPSINS OF GLOMERELLA RUFOMACULANS AND SPHAEROPSIS MALORUM<sup>1</sup>

By H S REED AND H S STAHL

(Received for publication, June 25, 1911)

Since the discovery of erepsins in the intestinal mucosa of animals by Cohnheim<sup>2</sup> and their presence in various plant tissues by Vines<sup>3</sup> those enzymes have not been widely studied in plants. In the few fungi which have been examined erepsins have been reported. Vines reported them in *Agaricus campestris* and in yeast. Bourquelot<sup>4</sup> reported a protease in *Aspergillus* and *Penicillium*, which, while not identified as such, was probably erepsin and Dox<sup>5</sup> demonstrated erepsin in *Penicillium camemberti*.

While the presence of erepsin has thus been demonstrated in a number of saprophytic fungi, it is believed that its presence has not previously been demonstrated in parasitic fungi. The results set forth in this note form a part of a piece of work on the relation of certain parasitic fungi on their host plants, and were obtained from pure cultures of two fungi which infest the apple. An additional point of interest lies in the fact that Vines found that the pulp of the apple contains neither trypsin nor erepsin, although the skin of the apple contains erepsin.

The first tests were made by cultivating the desired fungus for two months on Dunham's solution (contains 1 per cent peptone) and examining it for tryptophane.

<sup>1</sup> Paper No. 13 from the Laboratory of Plant Pathology, Virginia Agricultural Experiment Station, Blacksburg, Va.

<sup>2</sup> Cohnheim *Zeitschr f physiol Chem*, xxxiii, p 451, 1901.

<sup>3</sup> Vines *Ann of Bot*, xli, p 1, 1902, xvii, pp 237 and 597, 1903, xviii, p 289, 1904.

<sup>4</sup> Bourquelot *Compt rend soc biol*, xlv, p 653, 1893.

<sup>5</sup> Dox Bulletin 120, Bureau of Animal Industry, U S Department of Agriculture, 1910.

The fungi were grown in 200 cc Erlenmeyer flasks each containing 75 cc of fractionally sterilized Dunham's solution. After inoculation the flasks were kept at a temperature of 24° C in the incubator. At first the fungi grew slowly but eventually the mycelium formed a compact mat which entirely covered the surface of the liquid and produced spores. At the end of two months the liquid in the cultures of *Glomerella rufomaculans* was amber colored. It was filtered through paper and 10 cc samples taken in test tubes. Each was acidified with dilute acetic acid and bromine water added drop by drop. This caused the development of a deep violet color, which is regarded as a reliable test for tryptophane. The test was distinct in all samples although somewhat obscured by the amber color of the solution. The experiment was duplicated using two months old cultures of *Sphaeropsis malorum* which had been cultivated in the same way on Dunham's solution. Ten cubic centimeter samples were drawn into test tubes and received a few drops of acetic acid and bromine water. The filtered culture was nearly colorless and the deep violet color was very distinct. Control flasks of Dunham's solution of the same age as the cultures gave no tryptophane test.

The formation of this tryptophane from peptone by the growing fungus in pure culture is interpreted as evidence of the presence of erepsin in the fungus.

Supplementing the action of the erepsin-containing solutions upon peptone, studies were also made on the power of the intracellular enzymes of the fungi to liquefy gelatin. In these studies the fungus culture media were filtered from the mats of mycelium. After brief washing on the filters, the material was transferred to a porcelain mortar and ground with sand. This mass was extracted six hours with toluol water and filtered. The filtrate was placed in contact with gelatin, and at the same time an extract of cabbage was prepared and used in the same way.<sup>1</sup> Melted 7 per cent gelatin was placed in test tubes (3 cc in each tube) and received 3 cc, 5 cc, 10 cc, and 20 cc, respectively, of the extracts of cabbage, *Glomerella*, and *Sphaeropsis*. The tubes were held at 40° C for four hours and then

<sup>1</sup> For accounts of the ereptic activity of cabbage *vide* Dean *Bot Gazette*, *xxviii*, p 321, 1905, and Blood *this Journal*, *viii*, p 215, 1910

cooled The gelatin in every case failed to harden This was rather inconclusive on account of the large amounts of extracts used The experiment was repeated, using smaller amounts of the extracts, and 2 cc of 7 per cent gelatin in each tube The digestion was carried on eighteen hours at 40° C with thymol as anti-septic

*Digestion of gelatin by extracts of cabbage, Glomerella, and Sphaeropsis, after 18 hours*

AMOUNT OF EXTRACT ADDED	CABBAGE EXTRACT	GLOMERELLA EXTRACT	SPHAEROPSIS EXTRACT
cc			
0.3	Solid	Solid	Partially liquefied
0.5	Solid	Solid	One-third liquefied
1.0	Slightly liquefied	Slightly liquefied	One-half liquefied
2.0	One-half liquefied	Largely liquefied	Largely liquefied

These results show that the extracts of the mycelia of the two fungi employed were as capable of liquefying gelatin as the cabbage extract The results are, however, to be regarded as purely qualitative and do not permit of intercomparison of the different extracts Interest also attaches itself to the fact that erepsin was shown by Dean<sup>1</sup> to be principally if not entirely a metabolic enzyme rather than a digestive enzyme

The action of the fungus erepsin on casein was similarly studied and found to act positively

A two months old culture of *Glomerella rufomaculans* on Fermi's solution<sup>2</sup> was removed and washed with a stream of distilled water

The fungus mycelium was ground in a porcelain mortar, extracted fifteen hours with toluol water and filtered through filter paper The filtrate gave no test for tryptophane Two grams of casein was dissolved in 15 cc of  $\frac{N}{16}$  NaOH, diluted up to 60 cc and filtered into a small flask Fifteen cubic centimeters of the fungus extract were added to this and the flask incubated at 40° C with tol-

<sup>1</sup> Dean *loc cit*

<sup>2</sup> This solution contained distilled water, 1000 cc, magnesium sulphate, 0.2 gram, acid potassium phosphate 1.0 gram, ammonium phosphate, 10.0 grams, glycerol, 45 grams

uol present At the end of eighteen hours a marked tryptophane reaction was obtained The experiment was repeated using 20 cc of the fungus extract and incubating it for twenty-four hours A very strong test for tryptophane was then obtained

The fungus extract was not able however to produce tryptophane from protein A solution of 0.2 gram of "protein" (Eimer and Amend) was made in 10 cc of  $\frac{N}{10}$  NaOH and diluted to 50 cc in an Erlenmeyer flask Ten cubic centimeters of *Glomerella* extract filtered through paper were added with toluol and incubated at 40° C for twenty-four hours No test for tryptophane could be obtained

# STUDIES ON MELANIN III THE INHIBITORY ACTION OF CERTAIN PHENOLIC SUBSTANCES UPON TYRO- SINASE <sup>1</sup>

## A SUGGESTION AS TO THE CAUSE OF DOMINANT AND RECESSIVE WHITES

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(Received for publication, July 11, 1911 )

### INTRODUCTION

*The problem* An interesting biochemical problem is found in the study of the two kinds of white plumage and hair coloration which occur in the animal kingdom, and in the two forms of white which occur among the flowers. One form is common to all albinos and is uniformly recessive, *i e*, when crossed with a variety carrying color, all of the first generation offspring are colored. The other form of white, however, differs from the albino white in giving white offspring in the first generation when mated with color. This form is known as a dominant white. *We have, therefore, the fact that there are two forms of white, indistinguishable to the eye, and in so far as my chemical work has gone, indistinguishable to the chemist. Breeding experiments, however, show that these whites are exact opposites. That the difference between these whites must be comparatively slight is shown by the fact that there are a few instances known where a white is dominant to one color and recessive to the same color in another instance, providing that the two color crosses come from different individuals, breeds, or varieties.* For example, Dr Davenport has recently called to my attention the behavior of a flock of white ewes with which he has been conducting breeding experi-

<sup>1</sup>Reported in part before the Biochemical Division of the American Chemical Society at the Summer Meeting, Indianapolis, Indiana, June 28-July 1, 1911

ments at this Station. These ewes gave white offspring by one black ram and behaved in every respect as dominant whites. Later, however, a black ram of another breed produced black offspring when mated with these same ewes. Similar instances are known among the plants. While I do not claim to have proven the chemical cause of the behavior of dominant and recessive whites, I have been able to find some data which may apply to the problem and which furnish sufficient basis for a chemical explanation. The chemical study is being continued in this laboratory.

*Historical* The biochemical work which has been done on this problem is limited to a very few papers, the biological features, however, have been widely studied.<sup>1</sup> Miss Durham<sup>2</sup> obtained evidences of an oxidase in the foetal skins of rabbits and guinea pigs of black or agouti origin. She states that she is uncertain as to whether or not tyrosinase is present in the foetal skins of albinos but that there are indications that it is absent. No mention is made as to whether or not tests were made with the foetal skins of dominant whites. Pearson<sup>3</sup> in his monograph on "Albinism in Man" takes the view that pigments are the product of oxidase action and that the oxidase is probably lacking in albinos. Spiegler<sup>4</sup> in 1904 announced that he had isolated a "white melanin" from white horse hair and white wool. He makes no mention as to whether this material was of albinic origin or not, but the assumption is that it was of the dominant white variety, inasmuch as albinos are of rare occurrence among these animals. Spiegler's announcement interested biologists<sup>5</sup> in that it offered a possible explanation for the two forms of white, *i.e.*, the dominant white would be characterized by the presence of a "white melanin" while the recessive white would be recessive owing to the entire lack of pigment. Spiegler does not discuss this feature, but in a later paper<sup>6</sup> he suggests that this is a possible explanation.

<sup>1</sup> In this connection see Bateson *Mendel's Principles of Heredity*, The University Press, Cambridge, England, 1909.

<sup>2</sup> Durham *Proc Roy Soc*, LVIV, p 310, 1904.

<sup>3</sup> Pearson, Nettleship and Usher *A Monograph on Albinism in Man*, Part I, Drapers Company Research Memoirs, Biometric Series, VI, Dulau and Company, London, 1911.

<sup>4</sup> Spiegler *Beitr z chem Physiol u Path*, IV, p 40, 1904.

<sup>5</sup> Riddle *Biol Bull*, XVI, p 328, 1909, Spillman *Amer Nat*, XLV, p 119, 1910.

<sup>6</sup> Spiegler *Beitr z chem Physiol u Path*, X, p 253, 1907.

by saying, "One can readily understand that white horse hair cannot be without pigment. We know pigmentless hair, *i e*, *albinos*,<sup>1</sup> these have, apparently the natural color of the keratin modified by some special morphological condition. These questions need a more searching study."

In taking up a study of the melanins at this Station I repeated Spiegler's work and have already reported some of my results.<sup>2</sup> I will, however, include a summary of a portion of the work in this paper, inasmuch as the original article may not be readily accessible to chemists, and as it is from those results that I would reason

#### EXPERIMENTAL

*Spiegler's "white melanin"* In attempts to isolate this product, the keratin structure was boiled with sodium hydroxide solution of known strength for four hours. The mixture was then strained through cheese cloth and poured into cold water of sufficient quantity to at least double the volume of the alkali employed. This solution was strongly acidified with hydrochloric acid, throwing down a light grey precipitate. The supernatant liquid was decanted, the precipitate washed thoroughly by decantation, dissolved in 0.2 per cent sodium hydroxide solution, filtered, precipitated, washed by decantation, and the process of solution and reprecipitation repeated. The resulting product was dried on a water-bath, powdered, washed on a hardened filter with hot water, dried, extracted in a Soxhlet apparatus with carbon disulphide, alcohol and ether and dried at 105°. The weights obtained are shown in Table 1. Numbers 1 to 3 inclusive are from dominant white material, and numbers 4 to 8 are from recessive material in the Mendelian sense of the term.

It can be readily seen from this table that a "white melanin" does not exist in either variety of white, but that there is obtained from all forms of keratin, under the action of the alkali, a small amount of some substance which answers to the usual description of melanins, *i e*, is soluble in alkalis and insoluble in acids or neutral solvents. I have obtained similar products in like yields from fibrin and other proteins so that there can be no doubt that

<sup>1</sup>My italics. This statement of Spiegler's shows that his material was of the dominant white variety.

<sup>2</sup>Gortner *Amer Nat*, xlv, p. 497, 1910, *This Journal*, viii, p. 341, 1910.



TABLE 1

NO	SUBSTANCE	WEIGHT	VOLUME ALKALI	STRENGTH ALKALI	"MELANIN" FOUND	"MELANIN" FOUND
		grams	cc	per cent	grams	per cent
1	White wool	500	1675	10	0.30	0.06
2	White wool*	500	1675	10	0.30	0.06
3	White Leghorn feathers	46	200	10	0.09	0.19
4	White rabbit hair	100	350	10	0.03	0.03
5	"Silky" feathers	110	350	10	0.17	0.15
6	"Silky" feathers	105	700	5	0.24	0.22
7	"Silky" feathers	95	3000	1	0.16	0.17
8	Black wool	300	1000	10	7.35	2.45
9	Cows horn	40	200	10	Lost before weighing, but present in appreciable amount	

\*Had been previously digested at 40° for forty-eight hours with a solution of 18 grams of pepsin in 9 liters of 0.2 per cent hydrochloric acid

these substances do not belong to the true melanins. It is possible that they are related to the "anti-albumin" of Chittenden and Albro.<sup>1</sup> Indeed I have reasons for believing that most proteins contain a nucleus, which, under the proper conditions, may give rise to pigments.

*The anti-oxidase theory* Having shown that dominant whites do not contain a melanin which is lacking in the recessive whites, some other explanation must be sought for their different behavior.

Von Furth<sup>2</sup> has suggested that perhaps melanin is the product of an oxidase acting upon an oxidizable chromogen in the same manner that a black, insoluble, pigment precipitate is produced by the action of a tyrosinase on a solution of tyrosine. Accepting this theory, which has been proven true in a number of instances,<sup>3</sup> I suggested<sup>4</sup> that it was very probable that dominant whiteness was due to the presence of an inhibitory enzyme in the epithelial

<sup>1</sup>Chittenden and Albro *Amer Journ of Physiol*, 11, p 219, 1899

<sup>2</sup>v Furth and Schneider *Beitr z chem Physiol u Path*, 1, p 229, 1902,  
v Furth and Jerusalem *ibid*, 1, p 131, 1907

<sup>3</sup>Dewitz *Compt rend soc biol*, liv, p 44, 1902, Phisalix *ibid*, liv, p 19, 1905, Roques *Compt rend acad sci*, cxlix, p 418, 1909, Gortner *This Journal*, vii, p 365, 1910, Gortner *ibid*, 1, p 89, 1911

<sup>4</sup>Gortner *Amer Nat*, loc cit

cells which prevents the action of the oxidase, and that recessive whites differ in having neither the power to produce pigment due to lack of oxidase, or chromogen, or both, nor the power to inhibit its production when the elements for its formation are present. This being the case, *one form would be always dominant, its determiner being the anti-enzyme, and the other form would be uniformly recessive*. In making this suggestion I had no very clear idea as to the chemical nature of the anti-oxidase, but it now appears as though the inhibition of the pigment formation may be a purely chemical reaction, and not, necessarily, due to the presence of a true anti-enzyme. Perhaps the true solution lies somewhere between the two hypotheses and the inhibition may be chemical in some instances and due to enzymes in other cases. The new view, however, simplifies the matter, and would more readily explain those rarer cases in which the same white is both dominant and recessive, depending upon the cross.

Accepting the theory that all pigments are formed by the action of an oxidase on an oxidizable chromogen, and taking the specific example of tyrosinase and tyrosine, we find that when tyrosinase is added to a solution of tyrosine a series of color changes appears, passing through pink to red, and ending in the deposition of a black, insoluble, precipitate. *If, however, we add to the system, tyrosine + tyrosinase, a little orcin, resorcin, or phloroglucin, no coloration occurs.* This reaction can be only of three types

- 1 The *m*-dihydroxyl compound unites with the tyrosine to form some product which is not oxidized by tyrosinase
- 2 The *m*-dihydroxyl compound is more readily oxidized than is tyrosine, but gives colorless oxidation products
- 3 The *m*-dihydroxyl compound acts in the same manner as a true anti-oxidase and in some manner inhibits the action of the tyrosinase

I have found that the action is of the third type by eliminating both the first and second possibilities

- 1 If the colorless reaction mixture, tyrosine + tyrosinase + *m*-dihydroxyl compound, be washed on a filter with distilled water, a part of the tyrosine, on account of its slight solubility, remains on the filter, and on adding a small amount of fresh tyrosinase to this residue, the normal color changes are obtained. This shows that the tyrosine was not combined with the *m*-hydroxyl compound and eliminates type 1

2 That the reaction was not of type 2 was shown as follows, Seven test tubes were arranged in series containing respectively,

1	0.05 gm tyrosine
2	0.05 gm tyrosine + 0.05 gm orcin (1-methyl-3,5-dihydroxybenzol)
3	0.05 gm tyrosine + 0.05 gm resorcin ( <i>m</i> -dihydroxybenzol)
4	0.05 gm tyrosine + 0.05 gm phloroglucin (sym trihydroxybenzol)
5	0.05 gm orcin
6	0.05 gm resorcin
7	0.05 gm phloroglucin

To each tube was then added 0.02 gram of a very active preparation of tyrosinase from potatoes, prepared by repeated precipitation with alcohol and dried *in vacuo*<sup>1</sup>. Each tube was then half filled with water, a drop of chloroform added, and then tightly stoppered with a one-hole rubber stopper carrying a glass tube of 3 mm internal diameter, and bent twice at right angles so that the lower end of the tube dipped into water about 10 cm below the tube containing the reaction mixture. In this manner, if any oxygen were absorbed from the air in the stoppered tube, water would be drawn up in the glass tube and the height to which the water was raised would be a criterion of the amount of oxidation. No appreciable rise was noted in any of the tubes excepting nos 1, 3 and 6. The rise in nos 3 and 6 amounted to about 1 cm while the rise in no 1, which rapidly passed through the usual color changes, was nearly 10 cm. Apparently, therefore, orcin, resorcin and phloroglucin are not more readily oxidized than is tyrosine. That they are oxidized, and give colorless oxidation products, is improbable even if the above evidence were discarded, inasmuch as it has been shown that they are oxidized to *colored* compounds by other oxidases. Wolff<sup>2</sup> obtained an oxidase which is, apparently, as specific for the orcin type of compounds as tyrosinase is for tyrosine. I have also observed that laccase oxidizes orcin to a light red solution and resorcin and phloroglucin to orange solutions.

A series of tests was carried out using tyrosinases of various

<sup>1</sup> This tyrosinase contained a small amount of laccase which oxidized orcin to a pale pink at the end of ten days, and produced a yellowish coloration in the tubes containing resorcin and phloroglucin.

<sup>2</sup> Wolff *Compt rend acad sci*, cxlviii, p 500, 1909, *ibid*, cxlix, p 467

origins to determine whether or not the inhibition of the potato tyrosinase by the *m*-dihydroxyl compounds was peculiar to this variety of tyrosinase. Tyrosinase from the meal worm<sup>1</sup> (*Tenebrio molitor*), the Periodical Cicada<sup>2</sup> (*Tibicen septendecim* L.), and the mushroom, *Agaricus campestris*,<sup>3</sup> which I have found to contain both tyrosinase and laccase, were employed with the following results

*Experiments using tyrosinase from meal worms* To portions of this tyrosinase I added (1) tyrosine, (2) orcin, (3) phloroglucin, (4) pyramidon, (5) tyrosine + orcin, (6) tyrosine + phloroglucin, and (7) tyrosine + pyramidon. Tubes (1) and (7) were blue-black in two hours while all of the others remained colorless for ninety-six hours when they were discarded.

*Experiments using tyrosinase from the Periodical Cicada* In these tests I used the solution of the newly formed cuticula<sup>4</sup> which contained both the tyrosinase and the natural chromogen. To portions of this solution I added (1) nothing, (2) tyrosine, (3) tryptophane, (4) pyramidon, (5) phenolphthalein, (6) tincture of gum guaiac, (7) orcin, (8) phloroglucin, (9) resorcin, and (10) guaiacol. At the end of three hours numbers (1) to (5) inclusive were a dark smoky color, number (6) was deep blue, and number (10) was dark red brown. Numbers (7) to (9) inclusive were colorless. At the end of twenty hours the only change noted was that the smoky color in nos. (1) to (5) had become jet black and the blue of no. (6) was almost masked by the black which had developed. No trace of color had appeared in nos. (7) to (9).

*Experiments using tyrosinase (+ laccase) from Agaricus campestris* The tyrosinase, together with some laccase, was precipitated by saturating the expressed juice of the macerated mushrooms with ammonium sulfate, filtering, and dissolving in distilled water and again saturating with ammonium sulfate. The laccase could be prepared, free from tyrosinase, by precipitating the expressed juice with four volumes of alcohol, dissolving the precipitate in water and again precipitating with alcohol. The effect of the tyrosinase may also be removed by heating a solution

<sup>1</sup>Gortner *Trans. Chem. Soc.*, xcvi, p. 110, 1910.

<sup>2</sup>Gortner *This Journal*, x, p. 89, 1911.

<sup>3</sup>Identified through the kindness of Dr. Murrill and Mr. Seaver of the New York Botanical Garden.

<sup>4</sup>Gortner *This Journal*, x, p. 89, 1911.

of the two enzymes for one minute at 70°, whereby the tyrosinase is completely inactivated, while the activity of the laccase is not affected. Portions of the solution of tyrosinase (+ laccase) were treated as shown in Table 2

TABLE 2

TIME	ADDED CHROMOGEN	AFTER TWO HOURS	AFTER TWENTY HOURS
1	Tyrosine	Pink	Black
2	Tyrosine + resorcin	Unchanged	Orange
3	Tyrosol	Pink	Red
4	Tyrosol + resorcin	Unchanged	Orange
5	Gum Guaiac	Deep blue	Faded blue
6	Gum Guaiac + resorcin	Bluish	Bluish
7	Guaiacol		Dark brown
8	Guaiacol + resorcin		Pinkish yellow
9	Phenol		Dull red
10	Phenol + resorcin		Orange
11	<i>p</i> -Phenylenediamine	Deep red brown	Intense red purple
12	<i>p</i> -Phenylenediamine + resorcin	Unchanged	Greenish black
13	Enzyme one minute at 70° + tyrosine	Unchanged	Unchanged
14	Enzyme one minute at 70° + tyrosine and resorcin		Orange
15	Enzyme five minutes at 100° + resorcin		Unchanged

In this experiment the resorcin is oxidized by the laccase to an orange color, but the action of the tyrosinase is inhibited in every case by the *m*-dihydroxyl compound

On testing a solution of the laccase, prepared by alcoholic precipitation, it was found that it was without effect upon solutions of tyrosine or tyrosol, even in the presence of added hydrogen peroxide<sup>1</sup>. The oxidase, however, rapidly oxidized orcin to an orange-red and solutions of phloroglucin and resorcin to orange. Pyramidon was oxidized to orange-red, and solutions of cochineal were decolorized. In all of my experiments, checks, using a solution of the oxidase which had been heated for five minutes at 100°, were employed, and in no instance was the effect of oxidase action observed. These experiments were repeated in all

<sup>1</sup>Bach *Ber d deutsch chem Ges*, 31, p 221, 1908

sorts of combinations and the same general results were observed in each case

*The effect of o-dihydroxybenzol* A series of tests were carried out in which pyrocatechin was employed I found that pyrocatechin was readily attacked by tyrosinase (from potatoes), giving first a yellow solution which became red in the course of a few hours When tyrosine was added to the solution of pyrocatechin and tyrosinase the yellow coloration was the first to develop, but in a very few minutes this was completely masked by the red of the tyrosine oxidation The final black, however, was never reached Judging from this example, the benzol derivatives which carry two hydroxyl groups in ortho position to each other, are oxidized by tyrosinase to colored compounds, and do not inhibit the action of tyrosinase, although they may influence the course of the reaction Since both the ortho and para phenols are oxidized by tyrosinase to colored bodies, while the meta phenols are apparently not altered, it appears very probable that the colored oxidation products are of a quinoid nature

*General results* It would appear from these data, that aromatic compounds which carry two hydroxyl groups in meta position to each other, may act as *chemical anti-oxidases* on tyrosinase, and completely inhibit its action Other oxidases are not inhibited, but are able to oxidize these same *m*-dihydroxyl compounds, forming colored bodies of an unknown nature

#### CONCLUSIONS

I have shown that dominant whites do not contain a pigment which is lacking in the recessive whites I have also shown that aromatic compounds which carry two hydroxyl groups in the meta position to each other, are capable of inhibiting the action of tyrosinase on tyrosine or other chromogens, *even inhibiting the action of tyrosinase on the chromogens which produce the naturally occurring pigments*<sup>1</sup> Accepting, therefore, the theory that pigments are formed by the action of an oxidase on a chromogen, and taking, for example, tyrosinase and tyrosine, we have only to assume some reaction occurring in the animal body, whereby tyrosine is converted into either 3-hydroxyphenyl-

<sup>1</sup> See "Experiments using tyrosinase from the Periodical Cicada," above

$\alpha$ -aminopropionic acid, *i.e.*, the hydroxyl shifted from 4 to 3 or, else that an additional hydroxyl be added ortho to the alkyl chain, forming 2,4-dihydroxyphenyl- $\alpha$ -amino-propionic acid to secure a compound which should be incapable of pigment formation under the action of tyrosinase, and which should inhibit pigment formation even if both tyrosine and tyrosinase were present. Such a condition would produce dominant whites. The albinic whites could be explained as before by assuming that they lack either the enzyme, or chromogen, or both, and that they also lack the inhibiting factor which distinguishes the dominant whites. The theory of the chemical inhibitor would explain the rarer cases of recessive whites which are not albinic, and which are sometimes dominant. We have here only to assume the cross of tyrosine + *m*-dihydroxyl compound  $\times$  tyrosinase to secure dominant whites, *i.e.*, all of the first generation offspring would be white, while in the cross tyrosine + *m*-dihydroxyl compound  $\times$  some oxidase capable of oxidizing the meta compound, all of the first generation offspring would be colored, and the white would be recessive. Tyrosinase will not oxidize orein, and its action on tyrosine is inhibited by the presence of orein, which is, however, oxidized by a specific oxidase<sup>1</sup>. Tyrosinase will not oxidize pyramidon, (4-dimethylamino-1-phenyl-2,3-dimethyl-pyrazolon (5)), but the presence of pyramidon does not inhibit the action of tyrosinase on tyrosine. Pyramidon is itself oxidized by another specific oxidase<sup>2</sup>. These are only three examples of known specific oxidases so that it appears very probable that two breeds or varieties may differ in the nature of the pigment-forming enzyme<sup>3</sup>. We know of a number of specific enzymes in the different parts of the alimentary canal so that it is not improbable that the skin of the same animal may contain different oxidizing enzymes, or chromogens, in spots, so that the same white animal could possess both dominant and recessive white, in which case pied offspring would result from a cross with color.

<sup>1</sup>Wolff *loc cit*

<sup>2</sup>Bondouy *Trav Sci Univ Rennes*, 11, p 218, 1903

<sup>3</sup>This theory will also explain how white  $\times$  white sometimes gives colored offspring if we assume that the dominant white contains tyrosine + *m*-dihydroxyl compound + tyrosinase and the recessive white contains an oxidase capable of oxidizing the meta compounds but contains no chromogen. These two whites when crossed would produce colored offspring.

# MUCIC ACID AND INTERMEDIARY CARBOHYDRATE METABOLISM <sup>1</sup>

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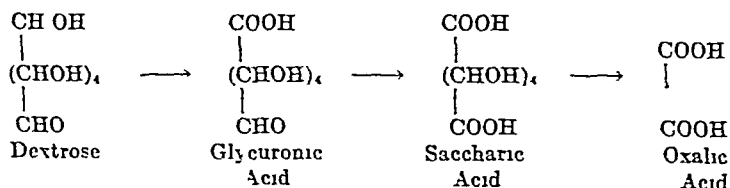
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(Received for publication, July 16, 1911 )

## INTRODUCTORY

Various views have been debated regarding the intermediate processes through which the carbohydrates pass in metabolism until they are finally eliminated in the form of their end products. As might be expected those theories fashioned from the experience gained in the study of the fermentation of sugars have received special attention <sup>2</sup>. Thus the formation of lactic acid from carbohydrates through the agency of microorganisms finds a counterpart in the occurrence of the same substance in the tissues and secretions of the animal body. Metabolism of the carbohydrate molecule by the mechanism here suggested, involves an early splitting of the carbon chain.

A distinctly different theory demands a direct oxidation of the carbohydrates without preliminary cleavage of the chain, with the formation of such intermediate products as glycuronic, saccharic and oxalic acids, as represented in the following formulas



<sup>1</sup>A preliminary report of this investigation was presented at the meeting of the American Society of Biological Chemists, December, 1910, see *Proceedings*, this *Journal*, ix, p vii, 1911

<sup>2</sup>The more recent aspects of this subject are reviewed by Harden *Alcoholic Fermentation*, 1911



This view was early supported by Schmiedeberg and Meyer,<sup>1</sup> who considered glycuronic acid a metabolic oxidative product of dextrose, on account of the related chemical structures of the two substances, and the ease with which dextrose is converted into glycuronic acid by oxidizing agents outside of the body. This view was also accepted by Emil Fischer and Piloty.<sup>2</sup> From feeding experiments with thymotimpiperidid, Hildebrandt<sup>3</sup> concluded that glycuronic acid may be formed from dextrose in the animal organism. He claims that fatal doses of this base, which conjugates with glycuronic acid, exert no toxic action in rabbits if the animals have previously received dextrose. According to P. Mayer,<sup>4</sup> the elimination of glycuronic acid may be markedly increased in severe disturbances of respiration, such as dyspnoea, and in diabetes mellitus. The observations of Mayer are, however, not substantiated by the more recent investigations of Fennyvessy,<sup>5</sup> and the statements on these questions are contradictory. That glycuronic acid is not a product of sugar metabolism seems to be indicated by the work of Loewi.<sup>6</sup> This investigator fed dogs with camphor during phlorhizin diabetes, and found that although enormous quantities of campho-glycuronic acid were excreted, the sugar elimination was only very slightly diminished. A much larger reduction in urinary dextrose ought to have occurred if it had been the mother-substance of glycuronic acid. Somewhat conflicting views in regard to this matter are given by Mandel and Jackson<sup>7</sup> and by Jackson.<sup>8</sup>

In experiments upon rabbits, Mayer<sup>9</sup> noted an increase in the output of oxalic acid after the introduction of glycuronic acid, either *per os* or subcutaneously. The livers of animals receiving glycuronic acid were found to be richer in oxalic acid than those

<sup>1</sup>Schmiedeberg and Meyer, H. *Zeitschr f physiol Chem*, III, pp 422-50, 1879

<sup>2</sup>Fischer and Piloty *Ber d deutsch chem Gesellsch*, LVII, pp 521-23, 1891

<sup>3</sup>Hildebrandt *Arch f exp Path u Pharm*, XLV, pp 278-316, 1900

<sup>4</sup>Mayer *Zeitschr f klin Med*, LVII, pp 68-108, 1902

<sup>5</sup>Fennyvessy *Arch internat d pharmacodyn*, VI, pp 407-20, 1904

<sup>6</sup>Loewi *Arch f exp Path u Pharm*, XLVII, pp 56-67, 1902

<sup>7</sup>Mandel and Jackson *Amer Journ of Physiol*, VIII, p VII, 1902-03

<sup>8</sup>Jackson *Amer Journ of Physiol*, VIII, p LVII, 1902-03

<sup>9</sup>Mayer *loc cit*

of control animals which had been similarly fed without glycuronic acid Autolytic experiments indicated that liver extracts contained enzymes capable of producing the change from glycuronic to oxalic acid

Thierfelder<sup>1</sup> has shown that saccharic acid results from the oxidation of glycuronic acid *in vitro*, but there is no direct evidence that this change can occur in the animal organism Mayer<sup>2</sup> reported an increase in urinary oxalic acid after subcutaneous injections of sodium saccharate The increase, however, is very slight Pohl,<sup>3</sup> after giving five grams of sodium saccharate to a small dog, was unable to detect any secondary oxidation products The urine was reported to contain neither saccharic acid nor an increase in oxalic acid On the other hand, Schott<sup>4</sup> in experiments on rabbits and dogs has recently found that saccharic acid is not oxidized in the body, but is excreted unchanged in the urine

After giving large doses of sugar, an increase in urinary oxalates has been observed in dogs by Baldwin,<sup>5</sup> and in rabbits by P Mayer<sup>6</sup> and Hildebrandt Baldwin interprets the oxaluria as due to a special kind of gastric fermentation, while Hildebrandt and Mayer consider it as indicative of an oxidation of sugar through the oxalic acid stage But, as Magnus-Levy<sup>7</sup> has pointed out, the extremely large doses of sugar (40 grams for a rabbit) may exert a toxic action leading to tissue disintegration which latter might give rise to the oxalic acid In fact there is considerable evidence indicating the possibility of oxalic acid being an intermediate product in the metabolism of the protein substance, or of the purines

As early as 1875, Fürbringer<sup>8</sup> claimed that diabetics excrete excessive amounts of oxalic acid, owing to decreased oxidative

<sup>1</sup>Thierfelder *Zeitschr f physiol Chem*, xi, pp 388-409, 1887  
Mayer *loc cit*

<sup>2</sup>Pohl *Arch f exp Path u Pharm*, xxxvii, pp 413-25, 1896

<sup>3</sup>Schott *Ibid*, lxxv, pp 35-7, 1911

<sup>4</sup>Baldwin *Journ of Exp Med*, v, pp 27-46, 1900-01

<sup>5</sup>Mayer *loc cit*

<sup>6</sup>Hildebrandt *Zeitschr f physiol Chem*, xxxv, pp 141-52, 1902

<sup>7</sup>Magnus-Levy *Oppenheimer's Handb d Biochem*, iv, Part 1, p 331, 1909

<sup>8</sup>Fürbringer *Deutsch Arch f Klin Med*, xvi, pp 499-526, 1875, xviii pp 143-92, 1876

functions, but Luzzatto<sup>1</sup> has been unable to observe any rise in the elimination. Indeed, it may be said that there is no convincing evidence supporting the assumption that glycuronic, saccharic, mucic, and oxalic acids are produced under physiological conditions by an incomplete combustion of carbohydrates.

Considering, however, the paucity of data in regard to the behavior and fate of mucic acid when introduced into the animal organism, and since if the unsplit-chain oxidation theory is correct one would expect mucic acid to result from the combustion of galactose, just as saccharic acid would result from the oxidation of dextrose, it seemed desirable to conduct experiments with mucic acid similar to those of P. Mayer with glycuronic and saccharic acids.

Baumgarten<sup>2</sup> administered 20 to 50 gram doses of the potassium salt of mucic acid *per os*, to normal and diabetic dogs and men, and was unable to recover any of the acid in the urine. From these experiments he concluded that mucic acid, in such quantities, is readily and completely oxidized in the body. The urines were not analyzed for possible oxidation products. Baer and Blum<sup>3</sup> found that the feeding of mucic acid is without effect on the ketonuria induced by phlorhizin diabetes. This indicates that if mucic acid is oxidized in the body, it is unable to replace carbohydrate as an acidosis-inhibiting agent. Baer and Blum also observed that mucic acid exerted a toxic action, resulting in the death of their animals. It is probable that this was due to the presence of impurities in their preparation. The crude product always contains toxic nitro-compounds, which are removed only after repeated recrystallization.

In the present investigation mucic acid was fed to rabbits and dogs, and the urines examined for mucic and oxalic acids. In the earlier experiments the mucic acid used was made from lactose by oxidation with nitric acid, according to the method of Kent and Tollens<sup>4</sup> and subsequently purified by recrystallization until the

<sup>1</sup>Luzzatto *Salkowski's Festschrift*, pp 239-52, 1904

<sup>2</sup>Baumgarten *Zeitschr f exp Path u Therap*, 11, pp 53-74, 1906

<sup>3</sup>Baer and Blum *Arch f exp Path u Pharm*, 14v, pp 1-32, 1911

<sup>4</sup>Kent and Tollens *Liebig's Annalen*, ccxvii, pp 221-32, 1885

resulting product was perfectly non-toxic and melted at  $213^{\circ}\text{C}$ . Later Kahlbaum's "Schleimsäure" was used with equally satisfactory results

## EXPERIMENTAL PART <sup>1</sup>

### *Methods*

The oxalic acid was estimated according to the method of Salkowski<sup>2</sup> which, from the result of comparative analyses made recently by MacLean,<sup>3</sup> seems to be less subject to error than the more commonly used method of Autenrieth and Barth<sup>4</sup>

It was impossible to determine the mucic acid excretion quantitatively, on account of want of an adequate method, but qualitative tests were made by oxidizing the urines with concentrated nitric acid, as Bauer<sup>5</sup> has proposed in testing for galactose

For this purpose, 100 cc -portions of the urine were introduced into beakers, each portion treated with 20 cc of concentrated nitric acid (sp gr, 1.4), and evaporated on the water-bath to a volume of approximately 20 cc<sup>6</sup>. The contents of the several beakers were then combined, transferred to a small crystallizing dish, further evaporated, and allowed to stand in a cool place over night. The pure white crystals were collected on a small filter paper, washed two or three times with water and alcohol, dried in a desiccator, and identified by the melting point. Preliminary tests showed that 0.2 gram of mucic acid, when added to 100 cc of urine, could be readily detected by this method. Five-tenths of a gram could be detected with the greatest ease. In one test where the latter quantity was added to 100 cc of urine, 0.26 gram, or over 50 per cent, was recovered after oxidation.

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<sup>1</sup>This investigation was undertaken at the suggestion of Professor Lafayette B. Mendel, and the experimental data are taken from the thesis presented by the author for the degree of Doctor of Philosophy, Yale University, 1911

-Salkowski *Zeitschr f physiol Chem*, xxix, pp 437-60, 1900

<sup>2</sup>MacLean *Ibid*, lx, pp 20-24, 1909

<sup>3</sup>Autenrieth and Barth *Ibid*, xxxv, pp 327-42, 1902

<sup>4</sup>Bauer *Ibid*, li, pp 158-66, 1907

<sup>5</sup>A flocculent, gelatinous, precipitate of silica usually separated when rabbits' urine was oxidized with nitric acid. This was removed by filtration before crystallizing the mucic acid

In testing for mucic acid in the feces, the following procedure was employed. The total fecal excretion for the period was rubbed up with water, the mixture made distinctly alkaline with sodium hydroxide, heated below boiling for five to ten minutes, and strained through absorbent gauze. The residue was in this manner extracted three times with water and alkali, the extracts combined, divided into 100 cc -portions, and oxidized with nitric acid, 20-30 cc of concentrated nitric acid being used for each 100 cc -portion of the extract. The total volume of the extract was 800-1000 cc for the excreta of a dog for two-day periods. In testing the delicacy of this method, it was found that half a gram of mucic acid when added to the dry's fecal discharge of a dog, could be almost quantitatively recovered.

The urines of the experimental animals were collected in periods of forty-eight hours. In the rabbits, the complete two days' excretion was obtained by squeezing out the bladders. With the dogs, no attempt was made to mark off the periods sharply.

Usually the animals were kept upon constant diets throughout the experiments, but occasionally they refused to eat as much during the experimental period as they did during the fore period. In such cases a corresponding reduction was made in the diet of the after period, so that in every case the urinary findings during the period of mucic acid feeding are comparable with those resulting from the same diet without the acid. The attempt to give the acid in the form of the neutral sodium salt almost invariably evoked diarrhoea, so that this method of administration was abandoned. It is probable that the large amount of sodium chloride formed in the stomach, through the action of the hydrochloric acid upon the sodium mucate, was responsible for the diarrhoea, by inducing a large secretion of water into the intestine. This explanation is rendered more probable inasmuch as doses of 5 grams of sodium chloride produce diarrhoea in rabbits. Hence, the rabbits received the free mucic acid suspended in water, by the stomach sound. With the dogs, the acid was mixed with the finely hashed meat and dog biscuit of the diet, and fed at regular intervals.

### *Rabbits*

The results of the nine series of experiments on rabbits are summarized in Tables I to III. In animals 1, 2, 3, and 4, the urine was not tested for mucic acid, the oxalic acid excretion alone being

TABLE I

RABBIT	DURATION OF PERIOD	VOLUME URINE	MUCIC ACID GIVEN	OXALIC ACID OUTPUT	DIET NOTES ETC
	days	cc	gms	mgms	
(1) 16 kilos	2	132	0	6.2	100 gms carrots per day
	2	100	10	13.4	100 gms carrots per day Mucic acid neutralized with NaOH, and given in two doses, 8 hrs apart
	2	200	0	7.5	100 gms carrots per day
(2) 21 kilos	2	420	0	10.2	250 gms carrots per day
	2	480	10	14.1	250 gms carrots per day Free Mucic acid given in 5 gm doses, 24 hrs apart
	2	320	0	9.0	250 gms carrots per day
	2	365	0	11.7	200 gms carrots, 25 gms corn, and 100 cc water per day
(3) 20 kilos	2	360	10	18.6	200 gms carrots, 25 gms corn, and 100 cc water per day Mucic acid given in two equal doses, 24 hrs apart
	2	375	0	5.9	200 gms carrots, 25 gms corn, and 100 cc water per day
(4) 22 kilos	2	525	0	9.1	500 gms carrots and 200 cc water during period

TABLE I—CONTINUED

RABBIT	DURATION OF PERIOD	VOLUME URINE	MUCIC ACID GIVEN	OXALIC ACID OUTPUT	DIET, NOTES, ETC.
	days	cc	gms	mgms	
(4) 2 2 kilos	2	255	15*	10 2	{ Ate only 355 gms carrots during period Given 200 cc water Mucic acid given in two doses -1st, 10 gms, 2nd, 5 gms, 24 hrs apart
	2	350	0	7 7	{ 355 gms carrots and 200 cc water during period

\*On evaporating the urine for the oxalic acid estimation, 0.1 gm mucic acid separated  
M.P. = 213° C

determined. In these experiments as in those on animals 6, 7, 8, and 9, there was only a slight increase in the oxalic acid output during the experimental periods, over that of the normal periods. The greatest percentage increase was noted in rabbit 7. Here, the output was 1.9 milligrams for the fore periods, 7.2 milligrams after receiving 10 grams, and 9.8 milligrams after receiving 20 grams of mucic acid. Even here the actual increase is extremely small—far less than one would expect if mucic acid were normally converted into oxalic acid in process of oxidation.

Part of the mucic acid was recovered unchanged in the urine in every case after giving doses as large as 15 grams, with the possible exception of rabbit 7. Here after 20 grams given in two doses of 10 grams each, only a trace of an organic precipitate was obtained, having the crystalline form of mucic acid.

In the periods when 10 grams of mucic acid were given, the unaltered acid was recovered only once in amount large enough to identify, and here the total quantity given was introduced in one dose. When the acid was given in two doses of 5 grams each, 24 hours apart, only a very small amount or none could be detected in the urine.

From the urine of animal 7, a trace of unaltered acid was recovered after a dose of 20 grams, while the urine of rabbit 8, a larger animal, yielded 0.2 gram of acid after a dose of 10 grams. Whether

TABLE II.

RABBIT	DURATION OF PERIOD	VOLUME URINE	MUCIC ACID GIVEN	MUCIC ACID RECOVERED	OXALIC ACID OUTPUT	DIET NOTES ETC
	days	cc.	gms	gms	mgms	
(5) 13 kilos	2	230	0	0		{ Constant diet of 150 gms carrots per day throughout the experiment
	2	340	15	0.5 (M.P. = 209°)		{ 1st day, 5 gms mucic acid 2d day, 10 gms free mucic acid
	2	345	0	0		
	2	250	0	0		
	2	225	0	0		{ 400 gms carrots and 50 gms corn during period
(6) 20 kilos	2	300	10	0		{ Ate only 300 gms carrots and 50 gms corn during period Mucic acid given in 2 equal doses, 24 hrs apart
	2	195	0	0		{ 300 gms carrots and 50 gms corn during period
	2	200	15	0.15 (M.P. = 209°)	3.9	{ Same diet as in preceding period 1st day, 10 gms free mucic acid 2d day, 5 gms free mucic acid
	2	250	0	0	3.4	{ Same diet as in preceding period



TABLE II—CONTINUED

RABBIT	DURATION OF PERIOD	VOLUME OF URINE	MUCIC ACID GIVEN	MUCIC ACID RECOVERED	OXALIC ACID OUTPUT	DIET, NOTES ETC.
	days	cc	gms	gms	mgms	
(7) 16 kilos	2	260	0	0	19	{ Constant diet of 200 gms carrots per day throughout experiment
	2	350	10	0	72	{ Mucic acid given in two equal doses, 24 hrs apart
	2	450	20	Trace*	98	{ 1st day, 10 gms free mucic acid 2d day, 5 gms free mucic acid
	2	450	0	0	54	

\* Very small precipitate was obtained which was identified as organic matter

this was due to individual variations in the ability to oxidize mucic acid, or to differences in rate and degree of absorption, could not be determined

### Dogs

Two experiments were made upon dogs Dog 10 (Table IV) was a small animal, and excreted relatively large amounts of the ingested mucic acid Throughout the experiment he was kept upon a constant diet, consisting of 100 grams of lean meat, 40 grams of cracker meal, and 25 grams of lard per day While the urines did not represent exactly 48 hour periods, the oxalic acid excretion agrees very well with the results obtained in rabbits Here also the actual increase during the mucic acid periods is slight and insignificant

In animal 11, a short experiment was made to determine whether or not any of the mucic acid failed to be absorbed, and was excreted in the feces The dog was fed 150 grams of meat, 100 grams of dog biscuit, and 30 grams of lard per day The feces were marked

TABLE III

RABBIT	DURATION OF PERIOD	VOLUME URINE	MUCIC ACID GIVEN	MUCIC ACID RECOVERED	OXALIC ACID OUTPUT	DIET NOTES ETC
	days	cc	gms	gms	mgms	
(8) 18 kilos	2	210	0	0	5.9	{ 400 gms carrots during period
	2	170	10	0.2 (M P = 212°)	6.9	{ Ate only 350 gms carrots during period Mucic acid given in one dose
	2	300	0	0	7.1	{ Same diet as in preceding period
	2	350	20	0.5 (M P = 213°)	8.2	{ Same diet as in preceding period Mucic acid given in two equal doses, 24 hrs apart
	2	450	0	0	7.9	{ Same diet as in preceding period
(9) 14 kilos	2	275	0	0	5.6	{ Constant diet of 150 gms carrots per day throughout experiment
	2	350	10	Trace*	8.5	{ Mucic acid given in two equal doses, 24 hrs apart
	2	350	0	0	6.4	
	2	400	15	0.5 (M P = 212°)	10.0	{ 1st day, 10 gms mucic acid 2d day, 5 gms mucic acid
	2	300	0	0	6.7	

Organic matter Melting point not determined

TABLE IV  
*Dog 10, 6.8 kilos*

DURATION OF PERIOD	VOLUME URINE	SPECIFIC GRAVITY	REACTION TO LITMUS	MUCIC ACID GIVEN	MUCIC ACID RECOVERED	OXALIC ACID OUTPUT	NOTES
<i>days</i>	<i>cc</i>			<i>gms</i>	<i>gms</i>	<i>mgms</i>	
2	500	1.020	acid	0	0	13.4	
2	250	1.032	acid	0	0	16.6	
2	300	1.032	acid	10	0.3 (M.P. = 210°)	25.1	{ Mucic acid was given in one dose
2	410	1.037	acid	20	1.3 (M.P. = 210°)	30.5	{ Mucic acid was given in two equal doses, 24 hrs apart
2	275	1.027	acid	0	0	7.8	

TABLE V  
*Dog 11, 13.2 kilos*

DURATION OF PERIOD	VOLUME URINE	SPECIFIC GRAVITY	REACTION TO LITMUS	MUCIC ACID GIVEN	MUCIC ACID RECOVERED IN URINE	MUCIC ACID RECOVERED IN FECES	NOTES
<i>days</i>	<i>cc</i>			<i>gms</i>	<i>gms</i>	<i>gms</i>	
1	176	1.050	alkaline	0	0	0	
2	300	1.046	acid	20	0	0	{ Mucic acid given in two equal doses, 24 hrs apart
2	250	1.027	acid	0	0	0	1st day of period
	170	1.025	alkaline	0	0	0	2nd day of period

off into two-day periods with lamp-black, and analyzed for mucic acid. The results are shown in Table V. After a dose of 20 grams, not a trace of the acid was recovered in the feces. Apparently, mucic acid is readily absorbed even when given in the free state. None was recovered in the urine of this animal after the mucic acid ingestion. The urine, which was alkaline to litmus for some

unknown reason during the fore period and last day of the after period, was rendered strongly acid for three days after giving the mucic acid. Whether or not the increased urinary acidity was due to a slight rise in the output of oxalic acid, or to the presence of traces of mucic acid too small to be detected by the method, was not determined.

Since rabbits and dogs—at least dog 10—excrete unaltered mucic acid in the urine after 20-gram doses, it seems scarcely probable that mucic acid is an intermediary product in the combustion of galactose and galactose-yielding carbohydrates. To further test this hypothesis, however, a series of experiments were made in which animals received the calculated amount of lactose (or galactose) necessary to yield 20 grams of mucic acid in the organism, and the urines were analyzed for mucic acid. The results follow.

EXPERIMENT 1 A rabbit weighing 2300 grams, received by the stomach-tube 35 grams of lactose, in two equal doses, twenty-four hours apart. Thirty-five grams of lactose, if oxidized through the mucic acid stage, should yield 21.4 grams of mucic acid. The animal was well fed on carrots and oats, and the urine collected for sixty hours. The urine contained *no sugar or mucic acid*.

EXPERIMENT 2 A rabbit weighing 1800 grams, received 35 grams of lactose in two equal doses, twenty-four hours apart. The urine excreted during the following fifty hours contained *no sugar or mucic acid*.

EXPERIMENT 3 A rabbit weighing 2020 grams, received two doses of lactose, 17.5 grams each, twenty-four hours apart. The urine of the following fifty hours contained *no sugar or mucic acid*.

EXPERIMENT 4 A rabbit weighing 2200 grams, received 8.6 grams of galactose. After an interval of twenty-four hours, a second dose of the same amount was administered. The total amount given (17.2 grams), if oxidized through the mucic acid stage, should yield 20 grams of mucic acid. The urine for the next sixty hours, contained a *minute trace* of sugar. No mucic acid was obtained after oxidizing with nitric acid. The amount of sugar present, if galactose, was too small to be detected by the Bauer method.

EXPERIMENT 5 A dog weighing 5.4 kilos, was given 35 grams of lactose in two equal doses mixed in with the food. The urine of the following fifty hours gave a slight reduction of Benedict's solution, no test with Fehling's solution, and yielded *no mucic acid* on oxidation with nitric acid.

EXPERIMENT 6 The same animal used in Experiment 5, received 35 grams of lactose in one dose. The urine of the following forty-eight hours gave a distinct reduction of Fehling's solution. The sugar was removed by fermenting with ordinary yeast (showing that the sugar was not lactose), the yeast filtered off, and the filtrate oxidized with nitric acid. *No mucic acid was obtained*.

EXPERIMENT 7 A dog weighing 6.2 kilos, received 35 grams of lactose in one dose. The urine of the following forty-eight hours contained a minute trace of sugar. Direct oxidation with nitric acid yielded no mucic acid.

#### GENERAL DISCUSSION

The results of the mucic acid feeding experiments, particularly those upon dogs, are not in accord with the findings of Baumgarten.<sup>1</sup> This investigator was unable to detect mucic acid in the urine of a medium size dog, after giving 20 grams, and in the urines of normal and diabetic men after giving 50 grams of the acid. The failure to detect mucic acid in the urine in any case, was probably due, at least in part, to the inadequate method employed, which consisted in evaporating the urine made alkaline with sodium hydroxide to dryness, extracting the residue with hot absolute alcohol to remove the urea, and preparing the double hydrazine compound of mucic acid,  $C_4H_4(OH)_4 (CO \cdot N_2H_2 \cdot C_6H_5)_2$ , by boiling on the water-bath with sodium acetate and phenylhydrazine-hydrochloride. Bulow,<sup>2</sup> who first prepared the compound, heated the mucic acid and phenylhydrazine on an oil-bath, at a temperature of 120° to 140° C. He states that the reaction is not complete until all water has been evaporated. Baumgarten seems to have overlooked these facts, although he cites the paper of Bulow in describing the method. It was found impossible to obtain the compound by Baumgarten's method of heating on a water-bath, even after the addition of 2 grams of mucic acid to the 100 cc. of urine used in the test. It seems scarcely probable that Baumgarten would have used the method without first testing its delicacy, but if such tests were made no mention of the fact is to be found in his paper.

In both the rabbit and dog experiments, the increase in oxalic acid excretion observed after giving mucic acid compares favorably in amount with that found by Mayer<sup>3</sup> after giving doses of 15 to 20 grams of sodium glycuronate or saccharate, and interpreted by him as indicating an oxidation of these substances to oxalic acid in process of metabolism. A similar interpretation is made

<sup>1</sup>Baumgarten *loc cit*

<sup>2</sup>Bulow *Liebig's Annalen*, ccxlvi, pp 194-97, 1886

<sup>3</sup>Mayer *loc cit*

of the increased oxalic acid excretion observed after giving rabbits 40-gram doses of dextrose, although the largest actual increase obtained was from 1.2 milligrams before, to 4.7 milligrams after the sugar administration. It would seem that the explanation of this small increase in oxalic acid suggested by Magnus-Levy<sup>1</sup> and already alluded to, is much more probable—namely, that the abnormally large doses of foreign substances produce a slight disintegration of body tissue, sufficient to occasion a rise in oxalate elimination.

It is not at all likely that oxalic acid is an intermediary product in the *normal* metabolism of any of the food-stuffs. Under certain abnormal conditions, at present little understood, it arises in the body, and is immediately excreted. This conception would seem to be indicated from the results of feeding experiments with oxalic acid. The inability of the organism to oxidize it, when introduced *per os* or subcutaneously, is conclusively shown by the researches of Gaglio<sup>2</sup> and Pohl.<sup>3</sup> Recently the latter investigator,<sup>4</sup> has corroborated his former work, having quantitatively recovered in the urine all the oxalic acid introduced into the organism. At any rate, no evidence has been obtained from feeding experiments with mucic, saccharic, and glycuronic acids, that oxalic acid is a normal intermediary product of carbohydrate metabolism.

Again, from the results of the lactose feeding experiments, the oxidation of the end carbon atoms of the sugar molecule without preliminary cleavage seems unlikely. In all experiments with the exception of experiment 6, (in which the lactose was given in one dose,) the sugar was completely utilized by the animals. Usually the sugar was given in two doses, 24 hours apart, in order to make the experiments exactly comparable with the mucic acid experiments. Twenty grams of mucic acid given in this manner, are not entirely oxidized by the body, but reappear in part in the urine. Stoichiometrically equivalent amounts of galactose-sugars, given under the same conditions, fail to evoke the appearance of mucic acid in the urine. The conclusion is obvious—*mucic acid is not a product of the physiological metabolism*

<sup>1</sup>Magnus-Levy *loc cit*

<sup>2</sup>Gaglio *Arch f exp Path u Pharm*, xii, pp 235-52, 1887

<sup>3</sup>Pohl *loc cit*

<sup>4</sup>Pohl *Zeitschr f exp Path u Therap*, viii, pp 308-11, 1910

*of galactose* It may be objected, that in the earlier experiments too much acid was introduced into the circulation at one time to allow complete oxidation, while in the latter series the change from lactose to mucic acid was very slow, only small amounts arising each moment. This explanation is, however, scarcely valid, inasmuch as mucic acid is relatively insoluble and its absorption must be very slow. Considerable time would be necessary for the content of the blood in mucic acid to be appreciably increased. Hence, there should be ample time for oxidation to occur, before the circulation becomes flooded with the acid. It is probable, therefore, that sugars are normally oxidized by some method other than that indicated by the unsplit-chain theory.

#### SUMMARY

1 Mucic acid, in doses of 10 to 20 grams, was not completely oxidized by rabbits, but was in part excreted unchanged in the urine.

2 When given in doses of 20 grams to a medium sized dog, mucic acid was excreted unchanged in the urine in amounts large enough to identify. The greater portion of the acid was not recovered.

3 After large doses of mucic acid, only a very small increase in oxalic acid elimination occurs in rabbits and dogs. This increase is by no means as large as would be expected if mucic acid were one of the precursors of oxalic acid.

4 Rabbits and dogs receiving the amounts of galactose and lactose stoichiometrically equivalent to 20 grams of mucic acid, excrete no mucic acid in the urine. Obviously therefore, *mucic acid is not an intermediary product in the metabolism of galactose-yielding sugars*.

# ON HYDANTOINS 1-PHENYL-2-THIOHYDANTOINS FROM SOME $\alpha$ -AMINO-ACIDS

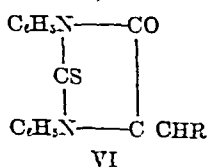
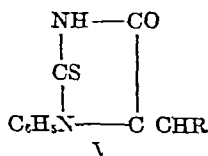
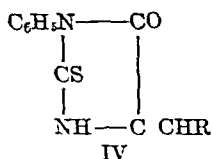
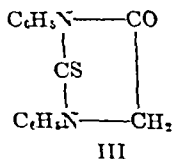
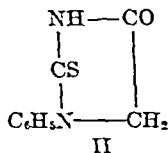
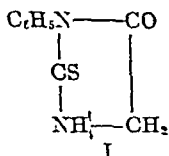
(Third Paper )

By CHARLES A BRAUTLECHT

(From the Sheffield Laboratory of Yale University )

(Received for publication, July 18, 1911 )

In a previous paper from this laboratory, Wheeler and Brautlecht<sup>1</sup> described the behavior of certain aldehydes towards 1-phenyl-2-thiohydantoin (I), 2-thio-3-phenylhydantoin (II), and 1, 3-diphenyl-2-thiohydantoin (III) They observed that the methylene hydrogens in these hydantoins are very reactive towards aromatic aldehydes, and that the three hydantoins condensed smoothly with aldehydes in glacial acetic acid, in the presence of anhydrous sodium acetate, giving the corresponding ethylene derivatives (IV, V and VI)



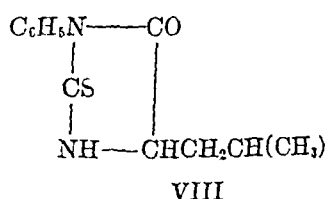
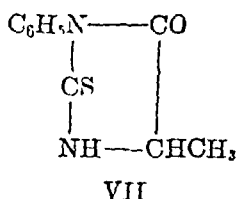
(R =  $\text{C}_6\text{H}_5$ -,  $\text{CH}_2\text{OC}_6\text{H}_4$ -, etc )

<sup>1</sup> Amer Chem Journ., xlv, p 446, 1911



It was necessary, in our study of the chemical behavior of some of these interesting condensation products, to first acquire a knowledge of 4-substitution products of 1-phenyl-2-thiohydantoin (I). In this paper I shall describe a series of such compounds, which I have obtained by the action of phenylisothiocyanate (phenyl mustard oil) on different amino-acids

2-Thio-3-phenylhydantoin (II), and 2-thio-1, 3-diphenylhydantoin (III) represent two new types of thiohydantoins and will be investigated later. Thiohydantoins of type I, however, have been known for some time. 1-Phenyl-2-thiohydantoin (I), was first prepared by Aschan<sup>1</sup> by the action of phenylisothiocyanate on glycocoll. He also investigated the action of this isothiocyanate on alanine and leucine, and obtained 1-phenyl-2-thio-4-methylhydantoin (VII), and 1-phenyl-2-thio-4-isobutylhydantoin (VIII), respectively. Aschan's method of operation was to melt together,



in a retort, the amino-acid and the isothiocyanate in molecular proportions, when the thiohydantoin was formed with separation of water. He observed however that the aromatic isothiocyanates only formed hydantoins smoothly in this manner, while the aliphatic mustard oils formed oily products, which could not be purified.

Marckwald, Neumark and Stelzner<sup>2</sup> later investigated the action of isothiocyanates on amino-acids and found that by a modification of Aschan's procedure it was possible to obtain crystalline thiohydantoins smoothly by the action of both aromatic and aliphatic isothiocyanates on amino-acids. They treated the potassium salts of the amino-acids in aqueous solution with the required amounts of the mustard oils when the potassium salts of the corresponding thiohydantoic acids were obtained in good yields. The latter on digestion with acids underwent condensation forming

<sup>1</sup> *Ber d chem Ges*, vi, p 1544, 1883, vii, p 420, 1884

<sup>2</sup> *Ibid* vii p 3278, 1891

the corresponding thiohydantoins They prepared in this manner, a series of 1, 4 substituted thiohydantoins by the action of phenyl-, *o* and *p*-tolyl-,  $\alpha$ -naphtyl- and allylisothiocyanates on a few amino-acids

With the exception of two recent papers by Bailey and Randolph<sup>1</sup> apparently no attention has been paid to phenylthiohydantoins since this work of Marckwald and his co-workers The only aliphatic  $\alpha$ -amino-acids so far examined are glycocoll alanine, leucine, and  $\alpha$ -aminoisobutyric acid<sup>2</sup>

I have now investigated the action of phenylisothiocyanate on  $\alpha$ -aminobutyric acid,<sup>3</sup> valine, phenylalanine, tyrosine, asparagine, aspartic acid and glutamic acid All these amino-acids reacted smoothly with this mustard oil, in the presence of alkali, forming the alkali salts of thiohydantoic acids, which readily underwent transformation into the corresponding thiohydantoins by treatment with hydrochloric acid Attempts to obtain crystalline phenylthiohydantoins from cystine and  $\alpha$ -pyrrolidonecarboxylic acid<sup>4</sup> were unsuccessful

The 1-phenyl-2-thiohydantoin derivatives of  $\alpha$ -amino-acids should be of service for the identification of these acids They are difficultly soluble in organic solvents and are well crystallized compounds, which have definite melting points Theyields were nearly quantitative in every case They undergo hydrolysis on warming with potassium hydroxide giving the potassium salts of the thiohydantoic acids While the corresponding oxygen acids are capable of isolation and are crystalline in character, the thio acids separate as oils when the alkali salts are treated with hydrochloric acid and undergo, at ordinary temperature, an inner condensation giving the original thiohydantoins They are desulphurized by digestion in aqueous or dilute alcoholic solutions with silver nitrate, mercuric chloride, lead oxide, mercuric oxide and bromine, and are stable in the presence of boiling hydrochloric acid The known 1-phenyl-2-thiohydantoins formed from  $\alpha$ -amino-acids together with their melting points and crystalline forms are given in the following table

<sup>1</sup> *Ber d chem Ges*, xli, pp 2494, 2505, 1908

<sup>2</sup> Bailey and Randolph *Loc cit*

<sup>3</sup> Fischer and Mounevrat *Ber d chem Ges*, xxxiii, p 2383, 1900

<sup>4</sup> *Zeitschr f physiol Chem*, lxi, p 457, 1910

TABLE I

AMINO-ACID	PHENYLTHIOHYDANTOINS	MELTING POINT	CRYSTAL FORM
Glycocoll <sup>*</sup> Alanine	1-phenyl-2-thiohydantoin*†‡	240-2°	plates
	1-phenyl-4-methyl-2-thiohydantoin*†‡	180-4°	prisms
$\alpha$ -Aminobutyric acid	1-phenyl-4-ethyl-2-thiohydantoin*	190-2°	plates
$\alpha$ -Aminoisobutyric acid	1-phenyl-4, 4-dimethyl-2-thiohydantoin§	174°	prisms
Valine	1-phenyl-4-isopropyl-2-thiohydantoin*	206-8°	needles
Leucine	1-phenyl-4-isobutyl-2-thiohydantoin*†	176-9°	prisms
Phenylalanine	1-phenyl-4-benzyl-2-thiohydantoin*	187°	prisms
Tyrosine	1-phenyl-4-hydroxybenzyl-2-thiohydantoin*	214-6°	prisms
Asparagine	1-phenyl-2-thiohydantoin-4-acetamide*	234°	needles
Aspartic acid	1-phenyl-2-thiohydantoin-4-acetic acid*	233-4°	lenticular prisms
Glutamic acid	1-phenyl-2-thiohydantoin-4-propionic acid*	169-70°	needles

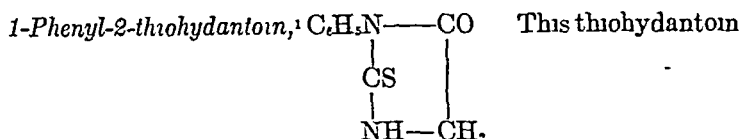
\*This paper †Aschan *Loc cit* ‡Marchwald et al *Loc cit* §Bailey and Randolph *Loc cit*

All of the above compounds are colorless except the 1-phenyl-2-thiohydantoin which, even after treatment with decolorizing agents and six recrystallizations from alcohol, still retains a pale straw color

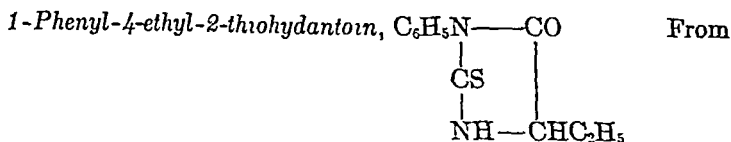
#### EXPERIMENTAL PART

*Preparation of Phenylthiohydantoins* The general procedure for preparing the thiohydantoins, described in this paper, was to dissolve the amino-acid, its hydrochloride, its ester or the ester hydrochloride in water containing the required amount of potassium hydroxide to form the potassium salt, and then adding the required amount of phenylisothiocyanate and a small amount of 95 per cent alcohol. The mixture was then digested on a sand bath, using a return condenser, until the reaction was complete. Frequent shaking hastened the reaction, and in most cases the mustard oil disappeared after boiling for a short time, usually less than one

hour The solution was then concentrated on a steam bath to remove the alcohol, an excess of hydrochloric acid added and the solution then evaporated to dryness The thiohydantoin usually separated in crystalline condition as the solution became concentrated After trituration with cold water to remove potassium chloride, quantitative yields of the pure hydantoin were almost always obtained



can be obtained according to the above procedure or by heating, at a high temperature, ethyl phenylthiohydantoate<sup>2</sup> This ester melts at 85° to a clear oil, which evolves alcohol at about 150° and then solidifies in the hot bath forming the thiohydantoin On heating in a capillary tube, this compound becomes greenish in color at about 220° and then melts at 240-2° to a dark oil with decomposition Aschan<sup>3</sup> and Marckwald<sup>4</sup> state respectively that the compound decomposes above 200° and 210° Phenylthiohydantoin dissolves in saturated sodium bicarbonate solution and is recovered quantitatively on addition of acids One part of the hydantoin dissolves in 9000 parts of water at 25° and in 252 parts of boiling water One part dissolves in about 45 parts of boiling alcohol It can be purified easily by crystallization from glacial acetic acid The hydantoin is not desulphurized by digestion with chloracetic acid in aqueous solution



phenylisothiocyanate and  $\alpha$ -amino-*n*-butyric acid<sup>5</sup> The yield was 97 per cent of the calculated This hydantoin is soluble in alkali, difficultly soluble in boiling water and dilute hydrochloric acid

<sup>1</sup> Aschan *Loc cit*, Marckwald, Neumark and Stelzner *Loc cit*

<sup>2</sup> Fischer *Ber d chem Ges*, xxxiv, p 433

<sup>3</sup> *Loc cit*

<sup>4</sup> *Loc cit*

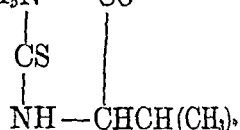
<sup>5</sup> Fisher and Mounexrat, *loc cit*

and moderately soluble in alcohol It separates from alcohol as square plates which melt at  $190-2^{\circ}$  It was dried for analysis at  $100^{\circ}$  (Kjeldahl)

N

Calculated for	Found
$C_{11}H_{11}ON_2S$	
12.36	12.36

*1-Phenyl-4-isopropyl-2-thiohydantoin*,  $C_6H_5N-CO$

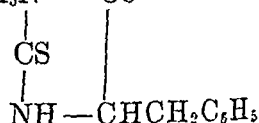


A quantitative yield of this compound was obtained by the action of phenylisothiocyanate on  $\alpha$ -aminoisovaleric acid (valine) It was purified by crystallization from alcohol, in which it is difficultly soluble, and separated as colorless needles melting at  $206-8^{\circ}$  to a clear oil It was dried for analysis at  $100^{\circ}$  (Kjeldahl)

N

Calculated for	Found
$C_{12}H_{13}ON_2S$	
11.97	11.93

*1-Phenyl-4-benzyl-2-thiohydantoin*,  $C_6H_5N-CO$

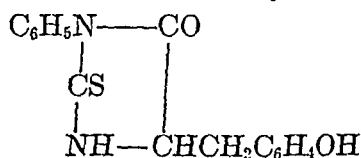


From synthetical phenylalanine and phenylisothiocyanate The hydantoin is readily soluble in acetone, benzene and glacial acetic acid, difficultly soluble in alcohol and practically insoluble in water It crystallized from alcohol as colorless prisms, which melt at  $187^{\circ}$  to a clear oil Analysis (Kjeldahl)

N

Calculated for	Found
$C_{16}H_{15}ON_2S$	
9.93	9.93

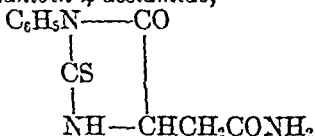
*1-Phenyl-4-parahydroxybenzyl-2-thiohydantoin*,



From one gram of tyrosine, which was obtained by hydrolysis of casein, I obtained by action of phenylisothiocyanate 1.54 grams of this thiohydantoin. The compound is soluble in glacial acetic acid, acetone and alcohol. One hundred grams of water dissolve 0.0789 gram of the hydantoin at 25°. It crystallizes from 95 per cent alcohol as colorless or straw colored prisms which melt at 214.6° to an oil. It was dried for analysis at 100° (Kjeldahl)

N	Calculated for	Found
	$C_{10}H_{14}O_2N_2S$	
	9.40	9.42

*1-Phenyl-2-thiohydantoin-4-acetamide,*



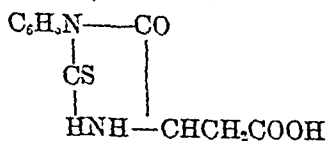
From asparagine (Kahlbaum's) and phenylisothiocyanate. This hydantoin crystallizes from alcohol as colorless lenticular prisms which melt at 234° to a clear oil. It is soluble in acetone and alcohol and insoluble in ether. One part of the compound dissolves in about 1550 parts of water. It is decomposed when warmed with alkali with evolution of ammonia. Analysis (Kjeldahl)

N	Calculated for	Found
	$C_{11}H_{15}O_2N_2S$	
	16.80	16.82

*Potassium Salt of the Hydantoinic Acid,*  $C_6H_5NHCSNHCH_2(COOK)CH_2CONH_2$ . This salt is readily soluble in water and moderately soluble in boiling alcohol. It separates from alcohol as colorless plates which melt at 154°. Analysis

N	K	Calculated for	Found
		$C_{11}H_{17}O_4N_2SK$	
		13.77	13.51
		12.79	12.83

*1-Phenyl-2-thiohydantoin-4-acetic acid*

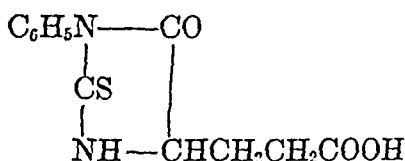


Two grams of 1-phenyl-2-thiohydantoin-4-acetamide were dissolved in 25 cc of water, containing two molecular proportions of potassium hydroxide, and the solution boiled until ammonia ceased to be evolved. The solution was then concentrated and acidified with hydrochloric acid. On evaporating the acid solution, the above hydantoin was obtained mixed with potassium chloride. The yield of the pure acid was 1.74 grams. It crystallizes from alcohol as colorless prisms, which melt at  $233-4^{\circ}$  with effervescence. One part of the acid dissolved in about 1747 parts of water at  $25^{\circ}$ . Analysis (Kjeldahl)

N

Calculated for $C_{11}H_{12}O_4N_2S$	Found
11.20	11.24

*1-Phenyl-2-thiohydantoin-4-propionic acid,*



Four grams of the hydrochloride of glutamic acid, which was obtained by the hydrolysis of gluten, were converted into the thiohydantoin in the usual manner. When the solution was cooled before the addition of the hydrochloric acid, the potassium salt of the corresponding hydantoic acid separated as glistening plates. This was not isolated. The hydantoin was purified by crystallization from alcohol and separated as microscopic needles which melted at  $169-70^{\circ}$  with slight effervescence to a clear oil. It is quite soluble in alcohol, difficultly soluble in boiling benzene and moderately soluble in ether. One part of the compound dissolves in about 556 parts of water at  $25^{\circ}$ . When an aqueous solution of the hydantoin is evaporated at  $100^{\circ}$ , a thick syrup is obtained, which shows very little tendency to crystallize. Analysis of the hydantoin after drying at  $110^{\circ}$

N

Calculated for $C_{12}H_{11}O_4N_2S$	Found
10.67	10.31

I acknowledge my indebtedness to Dr. H. L. Wheeler who suggested this work and to Dr. Treat B. Johnson for his assistance in the preparation of the results for publication.

# ON HYDANTOINS SYNTHESIS OF 3,5-DICHLOR- TYROSINE

(Fifth Paper )

PLATE II

BY HENRY L. WHEELER, CHARLES HOFFMAN AND TREAT  
B. JOHNSON

(Contributions from the Sheffield Laboratory of Yale University )

(Received for publication July 27, 1911 )

Since it is known that the halogens, chlorine, bromine and iodine, occur in living organisms in organic combinations, it seems plausible that they all may be linked in proteins in a similar manner. The identification of iodogorgonic acid, which Drechsel<sup>1</sup> isolated from the axial skeleton of a Gorgonian coral, as 3,5-diiodotyrosine<sup>2</sup> (I), and the recent isolation of this same substance from Florida coast sponges<sup>3</sup> and also from artificially iodized proteins<sup>4</sup> have therefore awakened an interest in the study of the corresponding chlorine and bromine derivatives of tyrosine (II and III). So far as the writer is aware, no chlorine- or bromine-containing organic compound of known structure has been isolated from animal tissues. 3,5-Dibromotyrosine (II) has been prepared by von Gorup<sup>5</sup>.

In this paper we shall describe the synthesis of the third member of this series, 3,5-dichlorotyrosine (III).

<sup>1</sup> *Zeitschr f Biol*, xxxiii, p 90, 1896

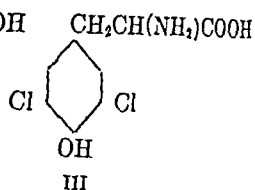
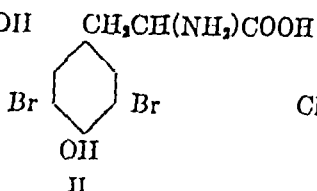
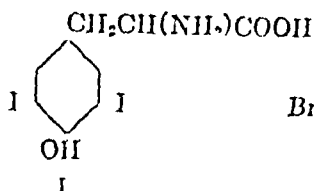
<sup>2</sup> Wheeler and Jamieson *Amer Chem Journ*, xxxiii, p 365

<sup>3</sup> Wheeler and Mendel *This Journal*, vii, p 1, 1909

<sup>4</sup> Oswald *Zeitschr f physiol Chem*, lxx, p 310, lxxi, p 200

<sup>5</sup> *Ann d Chem (Liebig)*, cxxv, p 281





The occurrence of chlorme in natural organisms was observed by Hundeshagen<sup>1</sup> in 1895, who detected its presence in certain species of corals. Drechsel<sup>2</sup> also found, a little later, that the skeleton of the Mediterranean coral, *Gorgonia Cavolinii*, from which he isolated iodogorgonic acid, also contained about 2.18 per cent of chlorme in organic combination. Henze<sup>3</sup> later made a thorough study of Drechsel's *gorgonin* and isolated, after hydrolysis of this substance with barium hydroxide, a small quantity of an apparently definite, halogen-containing compound. This possessed acid properties and contained 2.55 per cent of chlorine, but no nitrogen.

Mendel,<sup>4</sup> in 1900, determined the amounts of halogens present in three species of corals, which were collected in the West Indies, and observed the occurrence of chlorine in conjunction with iodine. For example—*Plexaura flexuosa*, *Gorgonia acerosa* and *Gorgonia flabellum* contained 0.86, 3.17 and 1.24 per cents of chlorine respectively. He did not however detect the presence of bromine. Morner<sup>5</sup> also examined several species of tropical corals and sponges and observed that they all contained not only iodine, but also chlorine and bromine in small amounts.

Panzer,<sup>6</sup> working with artificial halogen proteins, hydrolyzed chlorcasein with alkali and isolated a crystalline decomposition product, which was free from nitrogen, phosphorus and sulphur, but contained 62.8 per cent of chlorine. The compound was not identified, but Panzer states that it was possibly a highly chlorinated phenyl-propionic acid.

While 3,5-diiodotyrosine<sup>7</sup> (I) and 3,5-dibromotyrosine<sup>8</sup> (II) can

<sup>1</sup> *Zeitschr f angewandte Chem*, 1895, p. 473

<sup>2</sup> *Loc cit*

<sup>3</sup> *Zeitschr f physiol Chem*, LVIII, p. 60, 1903

<sup>4</sup> *Amer Journ of Physiol*, IV, p. 243

<sup>5</sup> *Zeitschr f physiol Chem*, LI, p. 33, 1907

<sup>6</sup> *Ibid*, LVIII, p. 131, 1901

<sup>7</sup> Wheeler and Jamieson *Loc cit*

<sup>8</sup> von Gorup *Loc cit*

be prepared easily by the action of iodine and bromine on tyrosine, all attempts to obtain a crystalline chlor-compound by the action of chlorine on the amino-acid have been unsuccessful Panzer,<sup>1</sup> for example, chlorinated natural tyrosine and obtained a product, which he states was similar in character to that obtained by hydrolysis of chlorcasein Wicke<sup>2</sup> states that when tyrosine was subjected to the action of chlorine water a resinous substance was formed, which was very soluble in alcohol No crystalline product was isolated

Aloy and Roberts<sup>3</sup> have recently examined the behavior of chlorine towards tyrosine and found that dry tyrosine is not attacked by this element On the other hand, they observed that chlorine reacted energetically with moistened tyrosine forming an indefinite, yellow-colored substance They state that this was chiefly a perchlororderivative, and was soluble in all common organic solvents Similar results were obtained by them when chlorine water was allowed to act on tyrosine or by treatment of the amino-acid in an acid solution with the gas

Since tyrosine does not react smoothly with chlorine, therefore efforts were made by us to obtain definite chlorine compounds by the action of this element on some derivatives of tyrosine We now find that tyrosinehydantoin<sup>4</sup> (V) reacts smoothly with chlorine in glacial acetic acid solution giving the corresponding hydantoin of 3,5-dichlorotyrosine (VIII) That the chlorine atoms had substituted in the 3,5-positions of the benzene nucleus was proved as follows The same dichlorhydantoin (VIII) was obtained easily by reduction of 3,5-dichlorobenzalhydantoin (VII) with hydriodic acid The structure of this benzal derivative (VII) is known It was prepared by Wheeler and Hoffman<sup>5</sup> by condensation of hydantoin (VI) with 3,5-dichlorobenzaldehyde<sup>6</sup> Therefore chlorine substitutes in the same positions (3,5) in the benzene nucleus of the tyrosinehydantoin, as are taken by iodine and bromine

<sup>1</sup> Loc cit

<sup>2</sup> *Ann d Chem (Liebig)*, ci, p 318

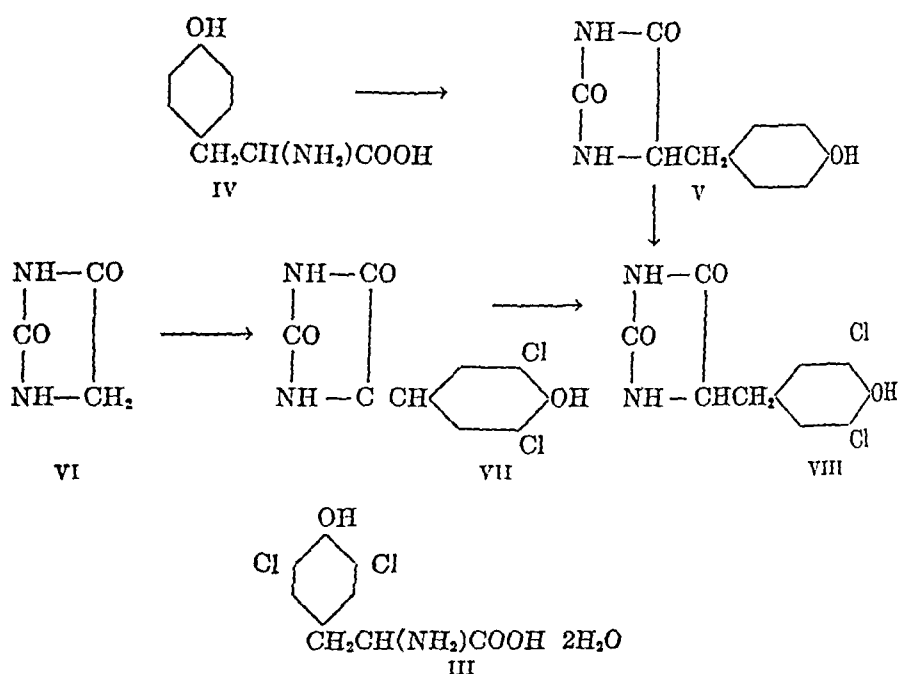
<sup>3</sup> *Bull soc chim*, iii, p 393, 1905

<sup>4</sup> Blendermann *Zeitschr f physiol Chem*, vi, p 253, 1882, Wheeler and Hoffman *Amer Chem Journ*, xlv, p 377, 1911

<sup>5</sup> Loc cit

<sup>6</sup> Luwers and Reis *Ber d chem Ges*, xxix, p 2356, 1896

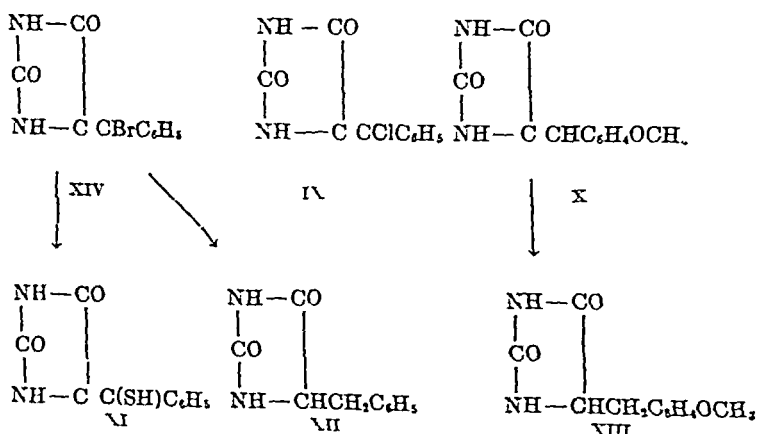
when they combine with tyrosine (IV) The chlorine atoms in 3,5-dichlortyrosine (III) are not removed by digestion of this compound with barium hydroxide solution The hydantoin however is unstable under these conditions, and undergoes a smooth hydrolysis, as has been observed in other cases, and is converted into the unknown 3,5-dichlortyrosine (III) These various transformations are represented by the following formulas



3,5-Dichlortyrosine (III) crystallizes like the corresponding dibromocompound<sup>1</sup> (II) with two molecules of water, while 3,5-dibromotyrosine (I) is anhydrous. It dissociates in aqueous solution and reacts acid towards litmus. It is precipitated from a dilute sulphuric acid solution by phosphotungstic acid. When heated with strong nitric and sulphuric acids it is decomposed with evolution of chlorine and hydrochloric acid. The acid gives no red color when warmed with Millon's reagent and forms insoluble silver, copper and lead salts. The salts with inorganic acids are extremely soluble in water.

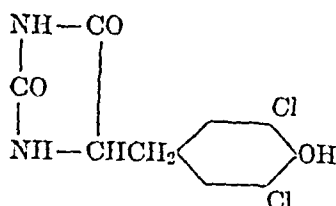
<sup>1</sup> Some new derivatives of this halogen amino-acid will be described in a later paper (T. B. Johnson)

In the course of our work we also examined the action of halogens on benzalhydantoin<sup>1</sup>. This compound is not attacked by iodine, but reacts with chlorine and bromine in glacial acetic acid solution forming  $\alpha$ -chlorbenzalhydantoin (IX) and  $\alpha$ -brombenzalhydantoin (XIV) respectively.  $\alpha$ -Brombenzalhydantoin is reduced by hydriodic acid forming smoothly benzylhydantoin (XII). This same hydantoin was also formed by reduction of benzalhydantoin with tin and hydrochloric acid. It is a remarkable fact that anisalhydantoin (X) was not reduced under practically the same conditions. By careful reduction of anisalhydantoin with hydriodic acid we succeeded in obtaining the corresponding anisylhydantoin (XIII). While the halogen in  $\alpha$ -brombenzalhydantoin (XIV) is easily removed by reduction with hydriodic acid, nevertheless this compound was extremely stable in the presence of reagents, with which it would be expected to react. For example, the bromine could not be removed by digestion of the hydantoin with potassium thiocyanate, potassium acetate, silver oxide, sodium ethylate or potassium hydroxide. On the other hand potassium hydrosulphide reacted smoothly giving  $\alpha$ -mercaptobenzalhydantoin XI, and potassium bromide



<sup>1</sup> Wheeler and Hoffman *Loc cit*

## EXPERIMENTAL PART

*Action of Chlorine on Tyrosinehydantoin 3,5-Dichlortyrosine hydantoin*

Five grams of tyrosinehydantoin were suspended in 30 cc of glacial acetic acid and heated at 100° on the steam bath. Chlorine gas was then conducted into the hot solution until the hydantoin dissolved. After evaporating to dryness a gummy residue was obtained, which we washed with ether when the greater portion of the material solidified. The weight of crystalline material was 4.7 grams and proved to be the dichlorhydantoin. The compound is very soluble in alcohol and glacial acetic acid and difficultly soluble in water. It crystallizes from alcohol in rhombohedral prisms, which melt at 202° with decomposition. It does not give Millon's reaction.

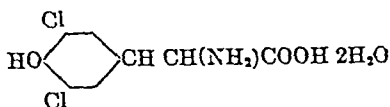
## ANALYSIS (Kjeldahl)

	Calculated for $\text{C}_{10}\text{H}_8\text{O}_4\text{N}_2\text{Cl}_2$	Found
N	10.18	9.75

*Preparation of 3,5-Dichlortyrosinehydantoin by Reduction of 3,5-Dichlor-p-hydroxybenzalhydantoin*<sup>1</sup>

Three and three-tenths grams of 3,5-dichlor-p-hydroxybenzalhydantoin were suspended in a mixture of 30 cc of glacial acetic acid and 15 cc of hydriodic acid, and then heated at 100° until the hydantoin completely dissolved. Iodine was evolved. After concentration to a small volume and saturation with sulphur dioxide gas to decolorize the solution, the dichlortyrosinehydantoin separated. It weighed 2.4 grams corresponding to a yield of 70 per cent of theory. It crystallized from alcohol in rhombohedral tables, which melted at 202°. A mixture of this reduction product with some dichlortyrosine from the preceding experiment melted at exactly the same temperature.

<sup>1</sup> Wheeler and Hoffman *Loc cit*

*3,5-Dichlortyrosine,*

Twelve grams of 3,5-dichlortyrosinehydantoin, 60 grams of crystallized barium hydroxide and 75 cc of water were digested in a flask attached to a return condenser for nine hours. The evolution of ammonia had then practically ceased. The solution was diluted with 300 cc of water, warmed to 100° and carbon dioxide passed into the solution to precipitate the barium as carbonate. After filtering from barium carbonate the filtrate was concentrated to a volume of 100 cc. This solution gave no test for chlorine when treated cold with silver nitrate and dilute nitric acid. On boiling this solution however a heavy precipitate of silver chloride deposited. The excess of barium was precipitated by addition of a little sulphuric acid, the barium sulphate filtered off, and the solution then concentrated until crystals began to separate on the surface of the liquid. On cooling we obtained 7.5 grams of dichlortyrosine, which melted at 248° with decomposition. After two crystallizations from hot water it melted at 252° with decomposition. This decomposition point varied according to the rate of heating. The amino-acid is sparingly soluble in water, four parts of the acid dissolving in about one hundred parts of the solvent. It is very difficultly soluble in alcohol, ether and benzene. When crystallized from water the acid separates in rectangular, prismatic crystals. It reacts acid in aqueous solution towards litmus and decomposes carbonates. It gives very soluble salts with hydrochloric and sulphuric acids, but is decomposed by cold, dilute nitric acid. Concentrated sulphuric and nitric acids decompose the acid with liberation of chlorine. It forms difficultly soluble silver and copper salts. The silver salt is soluble in dilute nitric acid. The acid is precipitated by phosphotungstic acid from dilute sulphuric acid solutions. It does not give Millon's test. It crystallizes with two molecules of water, part of which it loses when dried on a hot plate at 75-80°. The acid becomes anhydrous when heated at 110°.

## ANALYSIS

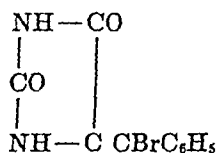
	Calculated for (C <sub>9</sub> H <sub>9</sub> O <sub>2</sub> NCl <sub>2</sub> ) 2H <sub>2</sub> O	Found
H <sub>2</sub> O	12.58	12.45
C	43.20	43.30
H	3.60	4.8
N	5.60	5.56
Cl	28.38	28.20

*Hydrochloride of 3,5-Dichlortyrosine* Five-tenths of a gram of dichlortyrosine was treated with 5 cc of dilute hydrochloric acid and warmed until dissolved. On cooling, large, prismatic, colorless crystals of the hydrochloride separated. It melted at 260–265° with decomposition.

## ANALYSIS (Kjeldahl)

	Calculated for C <sub>9</sub> H <sub>9</sub> O <sub>2</sub> NCl HCl	Found
N	4.81	4.95

*Reduction of Benzalhydantoin to Benzylhydantoin with Tin and Hydrochloric Acid* It has already been shown that this benzal compound can be reduced to benzylhydantoin by the action of hydriodic acid, and aluminium-amalgam.<sup>1</sup> Two grams of benzalhydantoin were dissolved in 95 per cent alcohol and 1.3 grams of tin added together with 20 cc of strong hydrochloric acid. The mixture was then heated on the steam bath until all the tin dissolved. The solution was evaporated to dryness and the residue triturated with water when 1.5 grams of benzylhydantoin were obtained. It melted at 190° to a clear oil, and a mixture of the compound and some hydantoin prepared from phenylalanine melted at the same temperature. Benzalhydantoin was recovered unaltered after long digestion in an ammoniacal solution with ferrous sulphate.

*α-Brombenzalhydantoin*

Five grams of benzalhydantoin were suspended in 25 cc of glacial acetic acid and 4.3 grams of bromine (Br<sub>2</sub>) slowly added

<sup>1</sup> Wheeler and Hoffman *Loc cit*

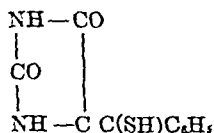
through a dropping funnel Heat was evolved and the benzalhydantoin dissolved without evolution of hydrobromic acid The solution was then evaporated to dryness on the steam bath Hydrobromic acid was slowly evolved during this treatment We obtained a crystalline residue which weighed 7 grams It was dissolved in alcohol and the solution cooled, when this benzal derivative separated in plates which melted at  $240^{\circ}$  to an oil The compound is moderately soluble in water and easily soluble in glacial acetic acid and alkali

## ANALYSIS (Kjeldahl)

	Calculated for $C_{12}H_{11}O_2N_2Br$	Found
N	10.48	10.75

*Reduction of  $\alpha$ -Brombenzalhydantoin to Benzylhydantoin with Hydriodic acid*

Five grams of  $\alpha$ -brombenzalhydantoin were heated on the steam bath with 15 cc of strong hydriodic acid and 30 cc of glacial acetic acid for four hours The solution became dark colored and iodine was liberated After concentrating the solution to a volume of 20 cc, water was added and sulphur dioxide gas passed in to reduce the free iodine An insoluble product separated here, which weighed 0.7 gram and was identified as unaltered material melting at  $240^{\circ}$  We separated from the filtrate 3.0 grams of benzylhydantoin, which melted at  $190^{\circ}$  to an oil

 *$\alpha$ -Mercaptobenzalhydantoin,*

Three grams of potassium hydroxide were dissolved in 25 cc of water and the solution saturated with hydrogen sulphide gas at  $0^{\circ}$  for one hour Five grams of the brombenzalhydantoin were then dissolved in the solution and the mixture boiled for one hour After cooling and acidifying with acetic acid we obtained 3.8 grams of the mercapto compound or 90 per cent of the calculated The hydantoin is very soluble in alcohol and glacial acetic acid and moderately soluble in hot water and ethylacetate It crystal-



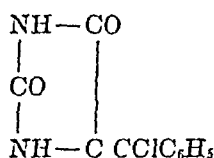
lizes from ethylacetate in yellow prisms, which melt at  $199^{\circ}$  with decomposition

ANALYSIS (Kjeldahl)

N	Calculated for	Found
	$C_{10}H_8O_2N_2S$	
	12.73	13.0

The halogen in  $\alpha$ -brombenzalhy dantoin is not removed by digestion with potassium thiocyanate, potassium acetate and silver oxide. Attempts to remove the halogen by heating with sodium ethylate and potassium hydroxide were also unsuccessful. The benzal compound underwent decomposition by the action of the two latter reagents giving a resinous substance which did not solidify. It was not examined further.

*$\alpha$ -Chlorbenzalhydantoin,*



Three grams of benzalhydantoin were dissolved in 300 cc of glacial acetic acid and the calculated amount (1 mol) of chlorine gas passed into the solution. Slight heat was evolved as the chlorine was absorbed. The solution was evaporated to dryness and the residue dissolved in alcohol. On cooling, the chlorcompound separated in plates, which melted at  $273^{\circ}$  to an oil. The hydantoin is very soluble in glacial acetic acid and difficultly soluble in hot water.

ANALYSIS (Kjeldahl)

N	Calculated for	Found
	$C_{10}H_7O_2N_2Cl$	
	12.58	12.35

Benzalhydantoin is not attacked by iodine. When the hydantoin was heated for several hours in glacial acetic acid with iodine no reaction whatever took place. The hydantoin was recovered unaltered.

*p-Methoxybenzylhydantoin,*

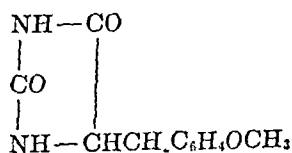




FIG 1 DIIODOTYROSINE



FIG 2 DIBROMOTYROSINE

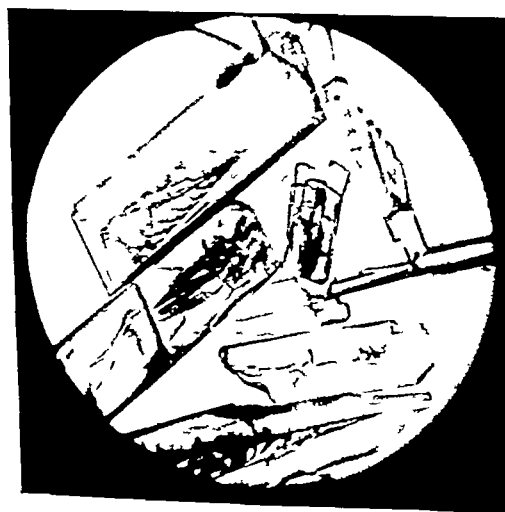


FIG 3 DICHLORTYROSINE



Five grams of anisalhydantoin<sup>1</sup> were partially dissolved in 75 cc of glacial acetic acid, 12 grams of strong hydriodic acid added, and the mixture heated at 100° for three hours. There was no evidence of the formation of any methyl iodide and iodine was set free. Sulphur dioxide water was then added to decolorize the solution, when 1.5 grams of the unaltered hydantoin, melting at 243°, separated. The filtrate was evaporated to dryness and the residue triturated with cold water, when we obtained 3.3 grams of *p*-methoxybenzylhydantoin. This compound is soluble in boiling water and alcohol. It crystallizes from water in yellow, hexagonal tables, which melt at 174° to a clear oil.

## ANALYSIS (Kjeldahl)

N	Calculated for	Found
	$C_{11}H_{12}O_3N$	
	12.73	12.97

An attempt to reduce this hydantoin with tin and hydrochloric acid was unsuccessful. This was remarkable, because the benzalhydantoin is reduced smoothly under similar conditions. Fifteen grams of anisalhydantoin were heated for one week, at 100°, with tin and concentrated hydrochloric acid. Some alcohol was also added to effect solution. Nothing but unaltered hydantoin was identified after this treatment. It was likewise recovered unaltered after digestion with zinc and glacial acetic acid. Five grams of the hydantoin were dissolved in 75 cc of glacial acetic acid, 35 cc of water and an excess of zinc added and the mixture boiled for three hours. After filtering and cooling, 4.2 grams of unaltered anisalhydantoin separated and melted at 243°. This product however was perfectly colorless while that obtained by condensation was always yellow and retained this color after repeated recrystallizations. A mixture of this colorless hydantoin and some of the yellow condensation-product melted at 243° proving that they were identical. When anisalhydantoin was digested for several hours with sodium hydroxide it was converted into *p*-methoxyphenylpyruvic acid melting at 185–7°.<sup>2</sup>

Photomicrographs of 3,5-diodotyrosine, 3,5-dibromotyrosine and 3,5-dichlorotyrosine are shown on Plate II. The three acids were crystallized from water.

<sup>1</sup> Wheeler and Hoffman *Loc cit*

<sup>2</sup> Wakeman and Dakin *This Journal*, ix, p 150, 1911



## STUDIES IN CARBOHYDRATE METABOLISM

### I THE INFLUENCE OF HYDRAZINE UPON THE ORGANISM, WITH SPECIAL REFERENCE TO THE BLOOD SUGAR CONTENT<sup>1</sup>

PLATE III

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New Haven, Connecticut)

(Received for publication, July 28, 1911)

The constancy of the sugar content of the blood under normal conditions constitutes one of fundamental axioms of physiology. It has been universally assumed that a certain supply of sugar in the blood is essential to normal metabolic rhythm and that even under distorted physiological conditions, as in inanition, the organism is capable of furnishing the requisite materials from its own economy for the maintenance of the blood sugar constant.

Attempts to upset this nice adjustment have resulted in the main in the temporary establishment of an excessive quantity of sugar in the blood. Thus hyperglycaemia may follow the introduction of various drugs into the body or may be produced by the induction of different pathological states. On the other hand, with the exception of phloridzin and uranium glycosurias, little has been known concerning the conditions necessary to diminish the content of blood sugar.

The practical significance of the investigations upon changes in blood sugar content obviously lies in the importance which the results obtained from such research may bear to the interpretation and treatment of human diabetes. It has been assumed that if the abnormal state which exists in diabetes could be experimentally reproduced, some hope for the prevention or alleviation of the condition might be realized. Such, however, has not been

<sup>1</sup>I am much indebted to Dr M S Fine for aid in carrying out some of these experiments

the case for, with the possible exception of pancreatic diabetes, an exact experimental duplication of the conditions existing in human diabetes has not yet been made

Recent investigations in the production of hypoglycaemia, notably those of Frank and Isaac,<sup>1</sup> with phosphorus, suggest a possible control of the blood sugar content which may lead to a distinct advance in our knowledge concerning certain phases of carbohydrate metabolism

The present paper is the first of a series in which it is planned to present various aspects of carbohydrate metabolism under conditions in which the sugar of the blood is experimentally diminished. The method employed for the production of this condition was the administration of the diamine, hydrazine

#### THE INFLUENCE OF HYDRAZINE UPON THE ORGANISM IN GENERAL

In their paper on "The Influence of Hydrazine upon Intermediary Metabolism in the Dog," Underhill and Kleiner<sup>2</sup> have described the general effects of hydrazine upon the organism in the following words

"The researches of Borissow,<sup>3</sup> of Pohl<sup>4</sup> and of Poduschka<sup>5</sup> have demonstrated the relatively great toxicity of this compound and have defined the series of manifestations following its introduction into the body. With doses of 0.1 gram hydrazine sulphate per kilo of body weight subcutaneously injected vomiting is observed which is succeeded by extreme restlessness. There is augmentation of the heart beat which later falls below the normal and respiratory difficulty is accompanied by general paralysis. At this stage a short period of coma usually ensues which terminates in death. The entire cycle of events is completed within a very few days. Coincident with the symptoms noted above is the appearance in the urine of varying quantities of protein and bile pigments."

From his histological study of the action of hydrazine, Wells<sup>6</sup> concluded that

"hydrazine seems to be a poison with an almost specific effect upon the cytoplasm of the parenchymatous cells of the liver, for when the poison is

<sup>1</sup>Frank and Isaac *Arch f exp Path u Pharmacol*, lxiv, p 274, 1911

<sup>2</sup>Underhill and Kleiner *This Journal*, iv, p 165, 1908

<sup>3</sup>Borissow *Zeitschr f physiol Chem*, xix, p 499, 1894

<sup>4</sup>Pohl *Arch f exp Path u Pharmacol*, xlviii, p 367, 1902

<sup>5</sup>Poduschka *Ibid*, xlv, p 59, 1900

<sup>6</sup>Wells *Journ of Exp Med*, x, p 457, 1903

given subcutaneously this tissue alone shows evident structural alterations, although equal or greater amounts must reach other organs or tissues. It seems to have remarkably little effect upon other than hepatic cells, and does not cause any appreciable destruction of red corpuscles, slight hemorrhages are occasionally produced, but much less than by other poisons with a similar effect upon the liver. It attacks only the cytoplasm of the liver cells, never affecting the nucleus primarily, and causes a profound fatty metamorphosis of the type commonly referred to as "fatty degeneration." In this respect it resembles phosphorus, from which it differs in two important particulars. Hydrazine attacks first the cells in the center of the lobules, while phosphorus shows its first and most marked effects upon the peripheral cells, and secondly, phosphorus usually causes marked fatty changes in the myocardium, the kidneys, and indeed throughout the body, whereas the effects of hydrazine seem to be limited almost absolutely to the liver. The unknown poisons of acute yellow atrophy and eclampsia, and most of the bacterial poisons, attack first and chiefly the nuclei of the liver cells, in contrast to the strictly cytoplasmic effects of hydrazine. Phosphorus also effects the nuclei more than does hydrazine. On this account the recovery of the liver to normal after hydrazine poisoning is remarkably rapid and complete, there being no permanent anatomical alterations after recovery from a most severe non-fatal poisoning."

Despite the grave injury to the liver it has been demonstrated that "the most striking feature of the action of hydrazine upon the animal body is the absence of abnormal relationships in the principal urinary constituents"<sup>1</sup> and the presence of abnormal substances, such as lactic, oxybutyric and diacetic acids, acetone or reducing bodies could not be detected in the urine, although in one instance there was a separation of a small quantity of cystine.<sup>2</sup>

Subsequent experience with hydrazine has afforded additional facts concerning the general influence of this poison upon the body of the dog which are of considerable importance for the conduct of future investigation. In the first place the subcutaneous administration of hydrazine sulphate in the dosage of 50 mgms per kilo presents an entirely different series of symptoms than a similar introduction of 100 mgms per kilo body weight. In both instances there is the same initial picture, *i e*, vomiting and extreme restlessness. With the smaller dosage, however, the animal appears merely drowsy and stupid during the first day. In general upon the second day the dog seems practically normal with the noteworthy exception that there may be evidence of

<sup>1</sup> Underhill and Kleiner *loc cit*

<sup>2</sup> Underhill and Kleiner *loc cit*



extreme weakness especially noticeable in the hind limbs. Food is refused. The animals may show a considerable loss of body weight, much more than can be accounted for by the few days starvation. Upon the fourth or fifth day food may be greedily eaten. After this stage is reached, the ultimate recovery of the animal is assured. In general nearly all dogs receiving the smaller dosage make complete recovery in spite of the fact that during the second and third day after the administration of hydrazine the liver presents, a typical, light colored appearance of "fatty degeneration." Bile may or may not appear in the urine and in general the urine contains no protein.

**METHODS** In the investigations here recorded, hydrazine (Kahlbaum) was always introduced subcutaneously as the sulphate in a 2.5 per cent solution which is a practically saturated solution at room temperature. Estimations of the blood sugar content were made according to the method indicated in a former paper,<sup>1</sup> the copper finally being determined gravimetrically. Glycogen in the liver was determined by Pflüger's method.<sup>2</sup> Blood pressure was recorded by a mercury manometer connected with a femoral artery. In blood pressure experiments narcosis was induced by a mixture of morphine and atropine (10 mgms morphine sulphate and 1 mgm atropine sulphate per kilo of body weight). Ether was not necessary after the preliminary operative procedures. Injections were made into the femoral vein.

#### THE ACTION OF HYDRAZINE UPON THE BLOOD SUGAR CONTENT

It has been previously intimated that there is a paucity of literature relating to the artificial production of hypoglycaemia. Aside from the consideration of the well known examples in connection with phloridzin and uranium salts, the experiments of Frank and Isaac<sup>3</sup> with phosphorus have shown for the first time the complete disappearance of sugar from the blood of rabbits. A decrease in blood sugar content has also been observed by Porges<sup>4</sup> after extirpation of the adrenals in dogs and in cases of adrenal insufficiency caused by Addison's disease. In all these instances the decreased sugar content of the blood is accompanied by a rapid decrease or complete disappearance of the liver glycogen in spite of the ingestion of relatively large quantities of carbohydrate.

<sup>1</sup> Underhill *This Journal*, 1, p 113, 1905-06

<sup>2</sup> Pflüger *Arch f d ges Physiol*, cxv, p 307, 1906

<sup>3</sup> Frank and Isaac *loc cit*

<sup>4</sup> Porges *Zeitschr f klin Med*, lxxv, p 341, 1909, lxx, p 243, 1910

What becomes of the glycogen of the liver and the sugar of the blood is at present largely a matter of conjecture. Whether the carbohydrate is merely transformed into some incompletely metabolized product and the latter eliminated, or whether more rapid metabolism is induced resulting in increased carbohydrate combustion remain problems for future determination.

**EXPERIMENTS WITH DOGS** Observations have been made to ascertain the influence of hydrazine upon the blood sugar content of dogs. From the data presented in Table 1 it may be seen that an appreciable hypoglycaemia follows the introduction of hydrazine in doses of 50 mgms per kilo of body weight, and that twice this quantity does not necessarily exert a more marked influence. In a large number of experiments hardly a single individual animal has failed to exhibit this phenomenon although variations in the degree of diminution may occur. The symptoms of extreme weakness in dogs after hydrazine treatment may be directly correlated with the diminished store of carbohydrate as indicated by lowered blood sugar percentage.

TABLE 1

*The Influence of Hydrazine upon Blood Sugar Content and Liver Glycogen of the Dog*

NUMBER OF ANIMAL	SUBCUTANEOUS INJECTION OF HYDRAZINE SULPHATE	SUGAR CONTENT OF BLOOD	GLYCOGEN CONTENT OF LIVER EXPRESSED AS GRAVE OF DEXTROSE	REMARKS
	<i>mgms per kilo</i>	<i>per cent</i>		
1	50	0.02	0.10	Three days after hydrazine injection
2	100	0.05	0.22	One day after hydrazine injection
13	50	0.03		Two days after hydrazine injection
15	50	0.04		Two days after hydrazine injection
A	0	0.14		Fasted six days
B	0	0.15		Fasted six days
C	0	0.13		Emaciated from lack of food

That starvation *per se* is not responsible for the lowered blood sugar content may be concluded from experiments A, B, and C, in which dogs were allowed to fast greater number of days than those in hydrazine experiments

**EXPERIMENTS WITH RABBITS** When hydrazine is administered to rabbits in doses of 50 mgms per kilo the animals refuse food for a period of at least two days. No other noteworthy symptoms are conspicuous. Since starvation plays a rôle in hydrazine experiments of this type it is imperative that the influence of this factor shall be determined. Accordingly determinations have been made of the sugar content of the blood and the glycogen of the liver in normal rabbits fed and kept under the usual laboratory conditions as a comparison for similar determinations made upon animals in every way comparable except that all food was withheld for a period of two days. This period was chosen for the reason that after hydrazine introduction a like period of time was allowed to elapse previous to killing the animals.

The results of these trials (Table 2) indicate what is generally accepted, namely, that the blood sugar content of normal rabbits is approximately 0.10 per cent, and that usually the liver contains a considerable quantity of glycogen although the quantity in individual rabbits may show a noteworthy variation. It is also evident that a period of two days inanition is sufficient to practically eliminate the liver's store of glycogen. On the other hand, the percentage of the blood sugar undergoes no appreciable change. When hydrazine is administered in the doses indicated it is clear that in the majority of cases the drug is capable of decreasing the sugar content of the blood, in some instances to a remarkable degree, in others only slightly, while a practically normal blood sugar content is maintained by a third group of individuals. From this diversity of results it is obvious that the rabbit can not be relied upon to invariably exhibit hypoglycaemia after hydrazine introduction. Hence results of experiments planned to supply data of carbohydrate metabolism from the standpoint of hyperglycaemia may be of questionable value.

The problem as to whether hydrazinized rabbits are capable of maintaining blood sugar content and glycogen store unchanged was subjected to experiment by subcutaneously introducing dex-

TABLE 2

*The Behavior of Blood Sugar Content and Liver Glycogen in Rabbits Treated with Hydrazine*

NUMBER OF ANIMAL	SUBCUTANEOUS INJECTION OF HYDRAZINE SULPHATE	SUGAR CONTENT OF BLOOD	GLYCOGEN CONTENT OF LIVER EXPRESSED AS GRAMS OF DEXTROSE	REMARKS
	mgms per kilo	per cent		
10	0	0 10	6 53	Well-fed rabbit
11	0	0 09	11 07	Well-fed rabbit
8	0	0 12	0 01	Two days fast
9	0	0 11	0 02	Two days fast
1	50	0 003	0 06	No food after hydrazine injection
2	50	0 016	0 00	No food after hydrazine injection
3	50	0 009	0 02	No food after hydrazine injection
12	50	0 08	0 00	No food after hydrazine injection
18	50	0 06	0 00	No food after hydrazine injection
19	50	0 09	0 00	No food after hydrazine injection
20	100			Died within five hours
16	100			Died within twenty-four hours
15	50	0 11		Subcutaneous injection 4 grams dextrose per kilo twice on day after hydrazine administration

trose into animals on the day following hydrazine injection. On the next day the animals were killed and the blood sugar content and glycogen of the liver were determined. The results of one such experiment (Rabbit 15, Table 2) are given. It is obvious from what has been said that the exact interpretation of these results is not easy since from the data furnished one is unable to know whether hydrazine exerted any appreciable influence upon this animal's carbohydrate store. It may be of inter-

est, however, to point out that in the two experiments, one of which is detailed in Table 2, Rabbit 15, carried out with this object in view both animals emitted sharp cries and exhibited a noticeable hyperpnoea immediately following the second injection of dextrose. This respiratory disturbance lasted about one minute, after which the animals appeared normal.

To determine the influence of small doses of hydrazine continued over a considerable period of time, two well-fed rabbits of 2100 and 2200 grams body weight respectively were selected and for twelve days each received 25 mgms of hydrazine sulphate daily without obvious detrimental influence of any kind. The only effect noted was an apparent ravenous appetite. After this period the dosage was increased to 75 mgms daily for eighteen days without any appreciable influence. Body weight had changed too little to be of significance. The animals were then killed. The blood sugar content of the two animals was 0.07 and 0.12 per cent respectively. The glycogen in the liver amounted to 1.56 grams and 5.1 grams expressed as dextrose.

In none of the hydrazinized rabbits was there any evidence of the characteristic light colored liver seen with dogs.

#### THE ACTION OF SUBCUTANEOUS INJECTIONS OF DEXTROSE UPON HYDRAZINIZED DOGS

Since hydrazine invariably causes a diminution in the percentage of blood sugar it was assumed that some light might be thrown upon the fate of the blood carbohydrate by a determination of the assimilation limits for dextrose injected subcutaneously into hydrazinized dogs. If dextrose of the blood undergoes a more rapid combustion in the body subsequent to hydrazine administration, one might conjecture that the organism would be capable of utilizing larger quantities of dextrose.

It has been shown that normal dogs completely utilize dextrose injected hypodermically in doses of 5 grams per kilo<sup>1</sup>. As hydrazinized animals refuse food it is essential to determine whether animals without food for a period of two days—the time selected

<sup>1</sup>Scott *Journ of Physiol*, xxviii, p. 107, 1902, Underhill and Closson  
This *Journal*, ii, p. 117, 1906

for sugar introduction subsequent to hydrazine injections—show as high assimilation limits as the normal animal

Three well-fed animals were selected for this purpose Dog 21 was a bitch of 5.1 kilos Dog 22 was a dog of 7.6 kilos Dog 24 was a hound of 15.7 kilos To these animals, after a preliminary inanition period of two days, subcutaneous injections of dextrose were given in doses of 5 grams per kilo The sugar was a 30 per cent solution For two days subsequent to the sugar injection, urine was collected and tested for dextrose In no case was there the least trace of reducing substance No food was given on the two days following the administration of dextrose

After a two days period during which the animals were well fed on a mixed diet, hydrazine was subcutaneously injected in doses of 50 mgms per kilo body weight On the second day following the hydrazine introduction quantities of dextrose were injected subcutaneously exactly equal to the amounts previously administered The sugar injections were all made in the late afternoon and when left for the night the animals were apparently in normal condition *The next morning all animals were found dead* The bladder of Dog 21 contained 13 cc of urine, 37 cc of urine were obtained from Dog 22, while the bladder of Dog 24 held 70 cc of urine In no case was there evidence of reducing substances Autopsy revealed nothing abnormal except the exhibition of the typical light colored liver

Three experiments in every respect similar except that the preliminary period of starvation and sugar injection was omitted yielded results exactly comparable All animals died within a period of twelve hours after the sugar injection The dosage of hydrazine alone was not sufficient to cause the death of the animals in this length of time, i e, within three days In fact, it has been our experience that with this dose, 50 mgms per kilo, practically all animals recover It is apparent that the combination of the two factors, hydrazine and dextrose, was necessary for the unexpected result

In some of the animals a noteworthy anuria was observed which may be of significance in the interpretation of the results obtained Why sugar solutions thus introduced should exert such a prompt toxic influence upon dogs treated with hydrazine remains to be ascertained This problem is being subjected to further study

## THE INFLUENCE OF HYDRAZINE UPON BLOOD PRESSURE

During the course of this investigation it became desirable to know the action of hydrazine upon the arterial blood pressure. That hydrazine exerts some influence in this respect might be concluded from the observation of Borissov<sup>1</sup> that hydrazine at first greatly increases the cardiac beat but subsequently causes a very significant slowing of the heart. Experiments to test this point have been made. A tracing (Plate III) is appended showing that even in relatively strong solutions hydrazine sulphate when introduced directly into the circulation fails to exhibit any immediate significant influence upon arterial blood pressure.

In this experiment a 10 kilo dog was employed. 15 cc of 0.1 per cent, 0.5 per cent, 1.0 per cent, and 2.5 per cent hydrazine sulphate solution were injected successively. The injections lasted about one minute. In the tracing time is recorded in half seconds.

## CONCLUSIONS

*Lethal Dose* Hydrazine sulphate subcutaneously injected into dogs and rabbits in doses of 100 mgms per kilo invariably culminates in death, while all animals usually recover from administration of 50 mgms hydrazine per kilo.

*Hypoglycaemia* Doses of 50 mgms per kilo hydrazine sulphate subcutaneously injected into dogs leads to a *distinct lowering of the percentage of sugar in the blood* with rabbits this effect is not constant.

During a short period of inanition, dextrose assimilation after hypodermic administration is as good as in normal well-fed dogs.

Dextrose in doses of 5 grams per kilo promptly causes death when subcutaneously injected into dogs previously treated with non-fatal doses of hydrazine.

Hydrazine introduced directly into the blood stream shows no appreciable immediate influence upon arterial blood pressure.

<sup>1</sup>Borissov *loc cit*







# NITROGEN FIXATION BY YEASTS AND OTHER FUNGI

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(Received for publication, July 28, 1911)

Since the epoch making discoveries of Hellriegel<sup>1</sup> there have followed in quick succession many series of investigations relative to the fixation of atmospheric nitrogen by living organisms. Especially brilliant are the investigations of Winogradsky<sup>2</sup> on *Clostridium pastorianum* and those of Beyerinck<sup>3</sup> on the *Azotobacter* group, and it is unnecessary here to go into a review of the investigations which have since added themselves to those, for the reader is doubtless acquainted with the painstaking and interesting researches of Gerlach and Vogel, Krüger and Schneidewind, J. G. Lipman, Löhnis, Christensen and a host of others to whom we are indebted for information on the subject of the fixation of atmospheric nitrogen by soil bacteria. Suffice it to say, that the researches of the past twenty-five years have disclosed facts which point emphatically toward the conclusion that the earth is endowed with agencies which, at least in part, compensate for the losses from our valuable nitrogen store from the soil which are constantly going on. From the earliest of these researches it appeared that the power of living organisms to assimilate atmospheric nitrogen was limited to a small group of bacteria of which the power seemed as characteristic and distinct as did the power of the group of nitrifying bacteria to change ammonium compounds to nitrates, but the later investigations soon brought to light the fact that many bacteria other than those of the *B. radicicola*, the *Azotobacter* and the *Clostridium* groups exhibited the power, more or less pronounced, of fixing atmospheric nitro-

<sup>1</sup> Ueber die Stickstoffnahrung der Gramineen und Leguminosen, 1838

*Compt. rend. de l'Acad. des Sci.*, 1893

<sup>2</sup> *Centralbl. f. Bakt.*, 17, 2 Abt., p. 3, 1902

gen These discoveries led further to a study of other organisms belonging to the groups of yeasts, molds and the higher fungi, in a search for those among them which possessed the peculiar physiological power to fix atmospheric nitrogen. These studies have resulted in both positive and negative results frequently with the same organisms in the hands of different investigators, and the present status of the question is still unsettled pending further evidence of the definite and constant powers of the fungi mentioned to fix atmospheric nitrogen. It was therefore with the double intention of making a further contribution to our knowledge of the nitrogen fixing powers of some of the organisms already studied as well as the study in that direction of organisms which until now, so far as the writer is aware, have not been experimented with, that the subjoined investigations were undertaken. It was decided to make a study of the nitrogen fixing powers of some of the true yeasts, "pseudo-yeasts,"<sup>1</sup> *Mycoderma* varieties and some of the common molds, among them *Aspergillus niger*, *Penicillium glaucum*, *Botrytis cinerea* and others. Before, however, going into the detailed description of the experiments carried out it will be helpful for purposes of comparison to review briefly the several researches, which have been carried out on the nitrogen fixing powers of organisms which are the same or similar to those employed in my experiments.

The investigations of Jodin<sup>2</sup> and Hallier<sup>3</sup> carried out as early as the sixties of the last century led them to believe that fungi were possessed of the power to fix nitrogen. Their results and opinions on this matter were not confirmed, however by Woff and Zimmerman<sup>4</sup>.

Sestini and del Torre reported some investigations in 1876 which seemed to have a doubtful significance. In their communication they make mention, however, of a statement by Selmi in which the latter attributes to the *Mycoderma* forms the power to produce ammonia from atmospheric nitrogen in the presence of nascent hydrogen.

<sup>1</sup> A term employed by zymologists to designate the yeast-like organisms which do not form spores.

<sup>2</sup> *Compt rend de l'Acad des Sci*, lv, p 612

<sup>3</sup> *Zeitschr f Parasitenkunde*, 1, p 129

<sup>4</sup> Abstract in *Jahresbericht der Agrikultur Chemie*, xiii-xv, p 169

The investigations of Loew and Nageli<sup>1</sup> led them to make the statement that elementary nitrogen is an unsatisfactory source of that element for molds Lawes, Gilbert and Warrington<sup>2</sup> could not show any fixation of nitrogen by some soil molds since analyses of soil, in which these molds were, showed no increase of nitrogen

Winogradsky has reported negative results in experiments with an *Aspergillus* species, and Czapek<sup>3</sup> has likewise reported negative results with *Aspergillus niger* and criticised the results of Puriewitsch and Saida, especially the latter, on the ground that the amounts of nitrogen fixed may have been due to errors in the nitrogen determinations Gerlach and Vogel<sup>4</sup> reported negative results with a yeast experimented with by them

To all of these doubtful or negative results on the fixation of nitrogen by yeasts, molds and other fungi, should be added the results of investigations very recently carried out by Duggar and Knudson<sup>5</sup> at Cornell University in which it is claimed that no nitrogen fixation was observed, except to a very slight extent in cultures of *Aspergillus niger*

Opposed to these negative results we have the positive results of several investigators in which we find a description of marked powers of fixing nitrogen attributed to the yeasts and molds experimented with Berthelot<sup>6</sup> noted gains of nitrogen in cultures of *Aspergillus niger*, *Alternaria tenuis* and *Gymnoascus*, but only the cultures of *Alternaria tenuis* were pure Puriewitsch,<sup>7</sup> however, worked with pure cultures of *Aspergillus niger* and *Penicillium glaucum* and in addition to all other precautions to exclude bacteria from these cultures he added some phosphoric acid to his culture solution which latter consisted of 100 cc of water, 0.4 gram of monopotassium phosphate, 0.4 gram of calcium chloride, 0.2 gram of magnesium sulphate, 3 grams of tartaric acid, varying amounts of dextrose and small amounts of ammonium nitrate

<sup>1</sup>Sitzungsber d mathem physik Klasse d Akad München, v, p 280  
Journ Chem Society, Transactions, xlii, p 203

<sup>2</sup>Beitr z chem Physiol und Pathol, ii, p 559, 1902

<sup>3</sup>Loc cit

<sup>4</sup>Abstract in Science, Feb 3, 1911, of a paper read before the Botanical Society of America

<sup>5</sup>Chimie végétale et agricole, i, Paris, 1899

Ber d deutsch bot Ges, xiii, p 339, 1895

Gains of nitrogen were noted in all cultures. It is interesting however, to observe in his results, the large differences in the amounts of nitrogen fixed by the same organism in duplicate cultures. Saida<sup>1</sup> not only confirmed the results of Puriewitsch on *Aspergillus niger*, but showed that a distinct nitrogen fixing power was possessed by *Mucor stolonifer*, *Endococcus purpurascens*, and *Phoma betae*. No gains of nitrogen, however, were noted by the same investigator in cultures of *Acrostalagmus cinnabarinus*, *Monilia variabilis* and *Fusisporium moschatum*. A Koch<sup>2</sup> claims that he and other investigators in repeating the experiments of Puriewitsch and Saida could not obtain any fixation of nitrogen, but calls attention to the fact that his results must not be taken as proof of the questionable purity of the cultures used by Puriewitsch and Saida nor yet of any error in the work of the last named investigators but rather to a change in the character of the organisms in old cultures as indeed this has often been noted in cultures of *Azotobacter* which after long standing seemed to have only a feeble nitrogen fixing power. This is an interesting observation which agrees with a similar one made by the writer in the experiments described below.

Frank<sup>3</sup> showed distinct gains of nitrogen in work carried out with different species of *Penicillium*, amounting in one case to 3.5 mg of nitrogen in 65 cc of nitrogen-free sugar solution in an incubation period of ten months. Remy<sup>4</sup> also showed fixation of nitrogen by three out of twenty-five molds which he tested and among these was *Aspergillus niger* which fixed 10 mg of nitrogen on 20 grams of dextrose as a source of energy. To these must also be added the investigations of Ternetz<sup>5</sup> who found in working with five species of *Phoma* that the latter possessed a pronounced nitrogen fixing power, noting in one case a fixation of 22 mg of nitrogen per gram of dextrose. The same investigator noted gains of nitrogen also in cultures of *Penicillium glaucum* and *Aspergillus niger* amounting in the latter case to 2.71 mg nitrogen per gram of dextrose.

<sup>1</sup>Ber d deutsch bot Ges, LV, p 107, 1901

<sup>2</sup>Handbuch der technischen Mykologie, Jena, III, 1907

<sup>3</sup>Landw Jahrb, XXI, p 7, 1892, Bot Zeitung, II, p 146, 1893

<sup>4</sup>Verh d Ges deutsch Naturf und Aerzte, LXIV, I, p 221, 1902

<sup>5</sup>Ber d deutsch bot Ges, LVII, p 267, Jahrb f wissen Botanik, XLV, 1907, p 353-408

Gains of nitrogen were also noted by Heinze<sup>1</sup> in cultures of yeasts in their spore forming stages and in cultures of lichens in the gonidium form. There should also be mentioned here the experiments of Gerlach and Vogel<sup>2</sup> in which they found a mold to possess the power of increasing the nitrogen content of the culture from 2.8 mg to 5.1 mg. This increase is attributed by Gerlach and Vogel to experimental error, but, as Löhnis well remarks in reviewing this matter, the other data given in the same investigations do not support such a view.

In a series of very carefully conducted experiments in which every possible precaution was taken to prevent absorption of ammonia and amido compounds from the air by the culture solutions, Fröhlich<sup>3</sup> showed very considerable and definite gains of nitrogen in cultures of fungi obtained from their growths on dead parts of plants. These fungi belonged to the hyphomycetes, and one of them *Macrosporium commune* fixed on the average as much as 8.92 mg of nitrogen per gram of dextrose used. In addition to these results the same investigator confirmed the results of others above noted with respect to the nitrogen fixing powers of *Penicillium glaucum* and *Aspergillus niger*. Latham<sup>4</sup> further confirms the results of Berthelot, Puriewitsch, Saida, Remy, Ternetz, Fröhlich and others above mentioned, in his experiments with *Aspergillus niger*.

Of particular interest in connection with the writer's results are those of Zikes<sup>5</sup> who describes a torula form, isolated from laurel leaves and named by him *Torula wiesneri* which possesses a power of nitrogen fixation equal to 2.3 to 2.4 mg per gram of glucose used. The experiments of Löhnis and Pillai<sup>6</sup> show only a slight nitrogen fixing power for a torula form employed by them, but a larger fixation of nitrogen by *Dematium pullulans*.

<sup>1</sup>Centralbl f. Bakt., 2. Abt., p. 675, xii, p. 357

Beitr. z. chem. Physiol. u. Path., ii, 1907

<sup>2</sup>Jahrb. f. wiss. Bot., xiv, p. 256, 1907

<sup>3</sup>Bull. Torrey Bot. Club, xxxvi, p. 235, 1909

<sup>4</sup>Sitzungsber. Akad. Wien, math.-naturw. Kl., cxviii, p. 1031

<sup>5</sup>Löhnis and Pillai Centralbl f. Bakt., xx, 2. Abt., p. 799

## EXPERIMENTAL

The material tested in these experiments consisted of seven species of *saccharomyces*, six varieties of "pseudo-yeasts,"<sup>1</sup> one mycoderma (*Mycoderma vini*) and three molds. The cultures employed were kindly given to the writer by Prof F T Bioletti of the California Agricultural Experiment Station to whom I desire, here, to express my sincere thanks. The cultures were all examined microscopically and appeared to be pure cultures of the organisms named in the tables.

*Series I*

A culture solution<sup>2</sup> was prepared and distributed in 100 cc portions in 500 cc Erlenmeyer flasks and sterilized in the autoclave. Each liter of solution consisted of the following

- 15.0 grams mannite
- 0.2 gram dipotassium phosphate
- 0.2 gram magnesium sulphate
- 0.02 gram calcium chloride
- 3 drops of a 10 per cent solution of ferric chloride
- 1000 cc tap water

The solution was rendered slightly alkaline to phenolphthalein by means of sodium hydrate. The solutions were carefully inoculated to prevent contamination, by means of a sterile platinum loop and placed in the incubator at 26°-28° C for one month, after which they were transferred to Kjeldahl digestion flasks, 30 cc concentrated sulphuric acid added and boiled on the digestion shelf until frothing ceased<sup>3</sup>, then about 12 grams of a mixture of K<sub>2</sub>SO<sub>4</sub>, FeSO<sub>4</sub> and CuSO<sub>4</sub> (in the proportion of 10 to 1 to  $\frac{1}{2}$ ) were added and the digestion continued for another hour or more. When cool the digested solutions were transferred to copper distilling

<sup>1</sup> These organisms are described by Holm Bull No 197, Cal Expt Sta tion

<sup>2</sup> Used by Lipman and Brown New Jersey Agr Expt Station, Bulletin No 210

<sup>3</sup> The method for the nitrogen determination used is given in detail because it presents some new modifications which allow of rapid and accurate work, as shown by tests in experiments which will be published shortly

flasks, diluted to proper volume, an excess of lye added, also some powdered zinc to prevent bumping and the ammonia distilling over was caught in  $\frac{1}{10}$  HCl. The excess of the latter was titrated against  $\frac{1}{10}$   $\text{NH}_4\text{OH}$ , and the amount of nitrogen fixed calculated in milligrams. Several sterile controls were run with each series. The results of the first series follow

TABLE I

No	NAME	NITROGEN FOUND	NITROGEN FIXED
		mg	mg
1	<i>Saccharomyces apiculatus</i>	1 75	0 84
2	<i>Saccharomyces ellipsoideus</i> champagne	1 87	0 96
3	<i>Saccharomyces cerevisioe</i> , carlsbergensis	1 87	0 96
4	<i>Saccharomyces ellipsoideus</i> , Steinberg	1 09	0 18
5	<i>Saccharomyces cerevisioe</i> , Distillery R.	1 04	0 13
6	<i>Saccharomyces ellipsoideus</i> , Bioletti	1 01	0 10
7	<i>Mycoderma vini</i>	1 40	0 49
8	Pseudo yeast, Tulare No 46a	1 26	0 35
9	Pseudo yeast, Tulare No 46b	2 38	1 47
10	Pseudo yeast, Tulare No 45b	0 91	0 00
11	Pseudo yeast, Tulare No 28a	1 12	0 21
12	Pseudo yeast, Tulare No 26	1 26	0 35
13	Pseudo yeast, Tulare No 37	0 95	0 04
14	<i>Aspergillus niger</i>	1 40	0 49

Every culture seems to show an increase of nitrogen except nos 10 and 13, but it may also be probable that the amounts fixed in nos 4, 5, 6 and 11 may be within the limits of error, and possibly even nos 8 and 12 may be included in this list. Nevertheless, there is a distinct fixation of nitrogen in nos 1, 2, 3, 7, 9 and 14, or in six out of fourteen cultures, and it is interesting to note that of these six, three are true yeasts, one a mycoderma, one a pseudo yeast and one a mold. The largest amount fixed in this series was that in no 9 by the "pseudo yeast" called Tulare no 46b.

It would seem therefore that these results are a further confirmation of those of Saida, Puriewitsch, Ternetz, Fröhlich, Zikes and others above mentioned, and it is especially interesting to note that the organism in the series above given which showed the largest fixation of nitrogen is very much like the *Torula wiesneri*



with which Zikes worked and which fixed considerably more nitrogen

That fixation should further have taken place in cultures representing the various classes of organisms experimented with, confirms the writer in the belief which he has held ever since these investigations were begun that the power of fixing atmospheric nitrogen though perhaps not a universal one among the lower plants is yet a very widespread power among the fungi. The fixation of nitrogen by true yeasts is, so far as the writer is aware, the first one described as such in the literature on the subject and adds another class of organisms to the now rapidly growing list of those which seem to be possessed of that power. It must also be mentioned here that the culture solution used above while a very good one for organisms of the *Azotobacter* group is not necessarily a satisfactory one for the organisms studied and was only employed because of the fact that so many different organisms were used and further obviously that no information is available as to what constitutes a good medium for nitrogen fixation for each class of organisms tested. It is possible, for example, that the alkaline reaction of the culture solution which is so necessary to the fixation of nitrogen by *Azotobacter* may be rather a hindrance than otherwise to the nitrogen fixing powers of *Aspergillus niger* or even the yeasts. Despite that, however, we have evidence of a power of nitrogen fixation, more or less pronounced, in each of these classes of organisms

### *Series II*

Owing to the fact that the zymologist values dextrose so highly as a medium for fermentation by yeasts, it was decided to arrange a series like the preceding but to substitute dextrose for mannite. The solutions were distributed in 100 cc portions in 500 cc Erlenmeyer flasks as described in the first series, and after the requisite sterilization and cooling were inoculated from the beer wort cultures of the organisms tested. The incubation was carried out in the same manner as in the preceding series, after which the solutions were analyzed for nitrogen according to the modification of the Kjeldahl method above described. The results of the analyses follow

TABLE II

NO	NAME	NITROGEN FOUND	NITROGEN FIXED
		mg	mg
1	<i>Saccharomyces apiculatus</i>	3.37	0.92
2	<i>Saccharomyces ellipsoideus</i> , champagne	2.69	0.24
3	<i>Saccharomyces cerevisiae</i> , carlsbergensis	4.19	1.74
4	<i>Saccharomyces ellipsoideus</i> , Steinberg	3.43	0.93
5	<i>Saccharomyces cerevisiae</i> , Distillery R	3.99	1.54
6	<i>Saccharomyces ellipsoideus</i> , Bioletti	2.63	0.18
7	<i>Saccharomyces ellipsoideus</i> , Burgundy	2.59	0.14
8	<i>Mycoderma vini</i>	4.24	1.79
9	Pseudo yeast, Tulare No 46a	4.73	2.23
10	Pseudo yeast, Tulare No 46b	3.71	1.26
11	Pseudo yeast, Tulare No 45b	2.52	0.07
12	Pseudo yeast, Tulare No 28a	4.54	2.09
13	Pseudo yeast, Tulare No 26	3.50	1.05
14	<i>Aspergillus niger</i>	4.63	2.23
15	<i>Penicillium glaucum</i>	3.15	1.70

A glance at Table II shows the influence of the kind of medium on the amount of nitrogen fixed. Not only does dextrose allow of a much larger fixation of nitrogen by the same organisms which showed fixation in mannite solutions but it allows other organisms to fix nitrogen which showed but a small nitrogen fixing power or none at all in the other medium. This is a factor which cannot be overlooked in considering the practical phases of nitrogen fixation as related to the nitrogen supply for plants in the universe. Here again, it would appear, the pseudo yeasts are more efficient at nitrogen fixation, when showing that power at all, than the true yeasts. For *Aspergillus niger* we find dextrose to be far superior to mannite as a source of energy, and we find it to have a power of nitrogen fixation in the medium which corresponds closely to that exhibited by the same organism in the hands of other investigators above mentioned. *Penicillium glaucum*, too, manifests a definite power of nitrogen fixation in dextrose solutions and this again corresponds to the results obtained by other investigators whose work is above reviewed.

We find thus that at least eleven of the fifteen organisms above tested show more or less pronounced powers of fixing nitrogen. Owing to the higher content of nitrogen in the tap water used at this time and also the higher content of nitrogen in the lye and

acid used in the nitrogen determinations, we find a much higher blank here, but several blanks were analyzed and the close agreement between them showed the results above given to be trustworthy. It should be mentioned here that the sterile blanks in all these series were not merely analyzed from the original solution but were inoculated like the cultures, then sterilized and incubated side by side with the cultures for the same length of time. It is interesting to note here that the amount of visible growth cannot be directly correlated with the amount of nitrogen fixed, for several of the cultures which appeared to have made only a small amount of growth showed quite a considerable fixation of nitrogen. I presume that this has been observed by other investigators working on this problem and is probably due to the fact that some of the nitrogen compounds produced are soluble and diffusible and therefore give no visible evidence of their presence. It is hardly necessary to add here that despite the favorable constitution of the medium employed it is not nearly so favorable for the growth of the organisms tested as the beer wort in which the stock cultures were kept as can be noticed particularly in the cultures of *Aspergillus niger* and *Penicillium glaucum* where the membranes formed in the dextrose solution are very thin and light in color and the spore production much smaller, than in the beer wort cultures.

### Series III

As explained above the culture solutions employed in the preceding series were prepared with tap water and the necessary sugar and salts added. It appeared to the writer that a more rigid test of the power to fix nitrogen possessed by the organisms in question should be made. It seemed desirable to see if, like the nitrogen fixing bacteria of the *Azotobacter* group, they had the power to fix nitrogen in nitrogen-free solutions or solutions which are practically nitrogen-free. It was also thought desirable to test the comparative values of mannite and four of the sugars in such nitrogen-free solutions as sources of energy for the organisms. To that end solutions like the one described above were prepared but distilled water, free from ammonia, was substituted for tap water. The salts employed being chemically pure and used only in small quantities as noted, could not contain more than a trace

of nitrogen. The sugars employed besides mannite were dextrose, maltose, lactose and saccharose. These were all chemically pure and thus the solutions when made up could only contain traces of combined nitrogen.

A change in the method of preparing the cultures in addition to the above should also be noted here. Twenty, instead of 15, grams of sugar were added to each liter of solution. The latter was distributed in 50 cc portions in 250 cc Erlenmeyer flasks each of which therefore contained 1 gram of mannite or sugar. The inoculations and incubation were carried out as in the preceding series except that the cultures in the maltose, lactose and saccharose solutions were incubated for twenty-five days instead of one month as were all the others. The superior nature of the tap water as compared with the distilled water was seen early in the incubation period. The growth in the distilled water cultures was much slighter and this was particularly noticeable in the case of the molds. The results of the nitrogen determinations follow, all arranged in one table so that the various sugars may be readily compared.

The results in Table III show clearly that every one of the organisms tested possesses a power, more or less marked, of fixing atmospheric nitrogen. In some cases that power seems to be so slight indeed as to be negligible but in most cases it is very distinct and definite. The next striking fact which presents itself for consideration in an examination of the foregoing table is the great difference in the nitrogen fixing power, of the several organisms tested, in the different media. While mannite seems to have been the most favorable source of energy for the largest number of organisms tested, some of the sugars employed allowed of the fixation of nitrogen by organisms which did not fix any nitrogen at all in mannite solutions.

The highest amounts of nitrogen fixed were quite considerable and compare well with the amounts fixed by pure cultures of *Clostridium pastorianum* and some of the less vigorous species of the *Azotobacter* group. We find here again in the dextrose and lactose solutions a confirmation of the work of other investigators above mentioned with respect to *Aspergillus niger*, and in the mannite solutions with respect to *Penicillium glaucum*. While the amounts fixed are in most cases not as large in these distilled

TABLE III

o	NAME	DEXTROSE		MANNITE		MALTOSE		LACTOSE		SACCHAROSE	
		Nitrogen		Nitrogen		Nitrogen		Nitrogen		Nitrogen	
		found	fixed	found	fixed	found	fixed	found	fixed	found	fixed
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
1	<i>Saccharomyces apiculatus</i>	1 05		2 38	1 54	0 70		1 79	0 99	0 98	0 30
2	<i>Saccharomyces ellipsoideus</i> , champagne	1 78	0 66	0 84		0 50		0 66		0 98	0 30
3	<i>Saccharomyces cerevisiae</i> , carlsbergensis	1 88	0 76	0 98	1 40	0 90	2 00	0 73		0 77	0 14
4	<i>Saccharomyces ellipsoideus</i> , Steinberg	1 26	0 14	1 40	0 56	0 70		1 16	0 36	0 98	0 30
5	<i>Saccharomyces cerevisiae</i> , Distillery R <sub>2</sub>	1 26	0 14	1 20	0 28	0 70				1 12	0 49
6	<i>Saccharomyces ellipsoideus</i> , Bioletti	1 21	0 09	0 78		1 05	0 35	1 34	0 54	0 84	0 21
7	<i>Saccharomyces ellipsoideus</i> , Burgundy	1 12		2 10	1 26	0 35		3 54	2 74	1 05	0 42
8	<i>Mycoderma vini</i>	1 71	0 59	0 84		1 36	0 66	1 51	0 71	0 63	
9	Pseudo yeast, Tulare No 46a	1 21	0 09	1 16	0 32	0 70		1 05	0 25	0 70	0 07
10	Pseudo yeast, Tulare No 46b	1 24	0 12	3 78	2 94	0 94	2 40	0 97	0 17	0 84	0 21
11	Pseudo yeast, Tulare No 28a	1 40	0 28	0 70		1 58	0 88	0 84	0 04	0 84	0 21
12	Pseudo yeast, Tulare No 26	1 82	0 70	1 54	0 70	0 41		1 08	0 28		
13	<i>Aspergillus niger</i>	1 75	0 63	0 98	1 40	0 59		2 13	1 33	0 84	0 21
14	<i>Penicillium glaucum</i>	1 12		2 80	1 96	0 75	0 50	0 85	0 05		
15	<i>Botrytis cinerea</i>	1 26	0 14	3 22	2 38	0 70		0 63		0 63	

water media as in the tap water media, they are in many cases definite enough to remove any doubt that these organisms are possessed of the power of fixing atmospheric nitrogen, even if amounts below 0.3 mg nitrogen are to be attributed to experimental error which indeed the writer very seriously doubts. There were slight losses of nitrogen from some of the culture solutions which can hardly be explained in any other way than by attributing them to experimental error. It is very striking that organisms which in their natural habitats are accustomed to drawing their nitrogen supply from a plentiful store of that element

can be made to fix atmospheric nitrogen in nitrogen-poor or nitrogen-free solutions with one of the sugars or mannite as a source of energy. Maltose and saccharose do not seem to be nearly as well suited to the growth and development of the organisms tested as lactose, dextrose and mannite, the last seeming to be the most favorable when the organisms are considered as a whole. The largest fixation of nitrogen was 2.94 mg per gram of mannite fixed by Pseudo-yeast Tulare no. 46b. The next largest 2.74 mg by the Burgundy wine yeast per gram of lactose and the next largest 2.38 mg per gram mannite by *Botrytis cinerea*. Several other considerable amounts were fixed by other organisms in mannite and lactose solutions which two seem to be the best suited for the fixation of large amounts of nitrogen in distilled water cultures.

#### GENERAL DISCUSSION

A careful consideration of the data above given brings further confirmation of the work of other investigators to the effect that the power of fixing atmospheric nitrogen is possessed by many of the lower organisms which differ widely in their character. Though the amounts fixed by them, as shown above, are not as large as those fixed by *B. radicicola* in conjunction with the leguminous plants nor yet as large as those fixed by the more vigorous species of the *Azotobacter* group, they are none the less definite and considerable. To the list of organisms which can fix atmospheric nitrogen as shown by former investigations may now be added the true yeasts and the "pseudo yeasts," besides *Botrytis cinerea*, an organism whose parasitic nature would seem to have deprived it of any nitrogen fixing power whatever. This in itself is a very interesting and striking fact. The fixation of nitrogen seems to have been made easier for the organisms in tap water solutions than in distilled water solutions owing to the small amounts of combined nitrogen present in the former. The nitrogen fixed would, in many cases, seem to have been of a soluble nature since considerable fixation was often noted in solutions where the growth would not seem to indicate it. The conclusions of Duggar and Knudson<sup>1</sup> are therefore not supported by the investigations above described. An effort is now being made by the writer to carry

<sup>1</sup>Science, N. S., **xxiii**, p. 191, 1911

out some experiments on the fixation of nitrogen by fungi similar to those with which Duggar and Knudson worked and it is hoped that the results may be available for publication in the near future

The objections of Czapek to the work of Puriewitsch and Saida would not seem to be valid in view of the writer's experiments, since the method of nitrogen determination employed as above described has been carefully tested in my laboratory, allows of a close agreement between duplicate series of determinations, and has given most satisfactory results in other phases of microbiological work

#### SUMMARY OF RESULTS

1 Of eighteen organisms, including yeasts, pseudo yeasts and molds, tested nearly all show a more or less pronounced power of fixing atmospheric nitrogen

2 Tap water sugar solutions are better suited for nitrogen fixation by the organisms tested than distilled water solutions

3 Mannite and lactose solutions are far superior to dextrose, saccharose and maltose solutions for these organisms in distilled water, but dextrose is the best in tap water solutions Maltose is the most unsatisfactory

4 The highest amount of nitrogen fixed was 2.94 mg per gram of mannite by pseudo yeast Tulare no 46b in distilled water mannite solution

5 The results of other investigators with reference to the nitrogen fixing powers of *Aspergillus niger* and *Penicillium glaucum* are confirmed

6 *Botrytis cinerea*, a parasitic fungus, has been found for the first time, so far as the writer is aware, to possess a nitrogen fixing power

My thanks are due my former assistant, Mr J A McKeen, for valuable assistance in making the nitrogen determinations

## PHYTASE IN LOWER FUNGI

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(Received for publication, August 7, 1911)

The occurrence of an enzyme which catalyzes the cleavage of phytin into inositol and phosphoric acid was first noted in rice bran by Suzuki, Yoshimura and Takaishi.<sup>1</sup> These investigators found that when rice bran was suspended in water and left for several days at room temperature the greater part of the organic phosphorus went into solution, and at the same time phosphoric acid was formed at the expense of the organic phosphorus compound. The same change was also observed in the organic phosphorus compound present in the oil cake of *Brassica napus*. The increase in inorganic phosphorus was in both cases so striking as to suggest the possibility that an enzyme might be responsible for the reaction. This was proved beyond all question by extracting some fat-free rice bran with water, removing the phosphates by means of barium chloride, precipitating the enzyme with alcohol, and testing its activity *in vitro* upon a phytin solution. Both phosphoric acid and inositol were identified in this case as cleavage products of the phytin.

It is probable that where phytin occurs in plants it is usually accompanied by the enzyme phytase which under proper conditions effects the decomposition of this phosphatide. In fact, the enzyme seems to be more widely distributed in nature than phytin itself. McCollum and Hart<sup>2</sup> tested various animal tissues for phytase, and found that liver and blood contained this enzyme, although extracts of kidney and muscle did not. Despite the fact that inositol is a well known constituent of animal tissues, phytin has not yet been isolated from them.

<sup>1</sup>Bull. Coll. Agr. Tokio, vii, p. 495, 1907.

<sup>2</sup>This Journal, iv, pp. 497-500.



The lower fungi, which numerous investigations have proved to be such fruitful sources of all manner of enzymes, have not, to our knowledge, been examined with reference to their phytin-splitting power. One of us<sup>1</sup> has shown that *Aspergillus niger* has the power of utilizing quite a variety of organic phosphorus compounds, among them phytin, as sources of phosphorus, and suggested that a cleavage of the phytin probably occurs before assimilation takes place. Such a cleavage would of course be accomplished by means of an enzyme secreted by the mold. It remained to be ascertained whether these lower fungi actually do secrete a phytin-splitting enzyme which would manifest its activity in the presence of a suitable antiseptic, and whether the enzyme would be formed in the absence of an organic phosphorus compound from the culture medium.

The culture fluid used in these experiments was a slightly modified Raulin's medium. In order to reduce the amount of inorganic phosphorus that would appear in the control tests, the ammonium acid phosphate prescribed by Raulin was reduced to 0.02 per cent. The tartaric acid was likewise reduced to one-half the usual amount, since the particular organisms under observation were found to thrive more vigorously in a less acid medium. One hundred cubic centimeters of this culture fluid were placed in each of a series of 300 cc. Erlenmeyer flasks and sterilized in the autoclave. Three well known species of *Aspergillus* were inoculated from pure cultures, and cultures one and two weeks old, respectively, were used in testing the phytin-splitting power.

To distinguish between intra- and extra-cellular enzyme, the mycelium and filtered culture medium were tested separately—a procedure followed by one of us<sup>2</sup> in a previous investigation on the catalase of molds. The mycelium was washed with distilled water, dried between filter papers, then divided into two portions of equal weight. Both portions were ground to a fine paste with quartz sand, stirred with successive portions of water and decanted from the sand until a complete separation was effected. To one portion of the turbid liquid thus obtained, 20 cc. of a 1 per cent phytin solution were added, and the total volume made up to

<sup>1</sup> Dox, *This Journal*, v, p 77, 1911

<sup>2</sup> Dox, *Journ Amer Chem Soc*, xxxii, pp 1357-61

100 cc The other portion, to be used as a control, was diluted to 100 cc with distilled water The filtered culture fluid was likewise divided into two equal portions, carefully neutralized, and made up to 100 cc, 20 cc of the 1 per cent phytin solution being added to one portion, and water to the other In all cases toluene was used for an antiseptic

The enzyme solutions thus prepared were allowed to act for a period of two weeks at a temperature of 28-30° At the end of that time, enzymic action was arrested by the addition of hydrochloric acid to 0.2 per cent, and the inorganic phosphorus determined by the method of Forbes<sup>1</sup> In addition to control tests where the mold was allowed to autolyze, a check experiment was made with phytin solution alone, and the inorganic phosphorus there found was also deducted from that found in the solution containing enzyme and phytin The analytical data are given in the following table, where *a* represents inorganic P<sub>2</sub>O<sub>5</sub> in the mycelial extract, *b* the same in the filtered medium

Cultures one week old

SPECIES		ENZYME AND PHYTIN*	ENZYME ALONE	INCREASE
<i>a</i>	<i>Aspergillus niger</i>	0.03044	0.00130	0.02734
<i>b</i>	<i>Aspergillus niger</i>	0.04064	0.00012	0.03892
<i>a</i>	<i>Aspergillus fumigatus</i>	0.02946	0.00294	0.02692
<i>b</i>	<i>Aspergillus fumigatus</i>	0.00524	0.00012	0.00352
<i>a</i>	<i>Aspergillus clavatus</i>	0.02936	0.00346	0.02430
<i>b</i>	<i>Aspergillus clavatus</i>	0.02602	0.00018	0.02424

Phytin solution alone contained 0.00160 gm inorganic P<sub>2</sub>O<sub>5</sub>

*Cultures two weeks old*

SPECIES		ENZYME AND PHYTIN*	ENZYME ALONE	INCREASE
a	<i>Aspergillus niger</i>	0 03360	0 00256	0 02924
b	<i>Aspergillus niger</i>	0 04058	0 00043	0 03839
a	<i>Aspergillus fumigatus</i>	0 03244	0 00043	0 03025
b	<i>Aspergillus fumigatus</i>	0 03062	0 00043	0 02843
a	<i>Aspergillus clavatus</i>	0 03286	0 00572	0 02536
b	<i>Aspergillus clavatus</i>	0 00310	0 00034	0 00098

\* Phytin solution alone contained 0 00178 gm inorganic P O<sub>5</sub>

The above data show an unmistakable increase of inorganic phosphorus resulting from enzymic cleavage of the phytin. The greatest cleavage seems to have been effected by the enzyme from *Aspergillus niger*. In all cases the enzyme is present in both the intra- and extra-cellular form.

# THE DETERMINATION OF CALCIUM IN THE PRESENCE OF MAGNESIUM AND PHOSPHATES THE DETERMINATION OF CALCIUM IN URINE

By FRANCIS H. McCrudden

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(Received for publication, August 3, 1911)

A year ago I published the details of a very accurate method for the quantitative estimation of calcium and magnesium in the presence of phosphates and small amounts of iron<sup>1</sup>. In the development of the method, accuracy was the first consideration, simplicity and rapidity having been deemed of secondary importance. During the past winter, in the course of investigations in which many calcium determinations were necessary, a more rapid method appeared desirable. The new method, therefore, has been studied with a view to ascertain to what extent its details could be modified in the direction of ease and rapidity of execution without essential loss of accuracy. The present paper gives a report of these investigations. Part I deals with pure solutions, Part II with modifications advisable in the case of urine.

## PART I THE DETERMINATION OF CALCIUM IN THE PRESENCE OF MAGNESIUM AND PHOSPHATES

The method as previously published is carried out as follows

The solution is brought to a volume of about 100 cc. and two drops of alizarin red are added. Ammonia is then added drop by drop until the red color of the alizarin just disappears (or, in the case of urine, until a precipitate of phosphate just appears). Dilute hydrochloric acid is then added drop by drop until the red color of the alizarin just reappears (or, in the case of urine, until the precipitate of phosphate just disappears). If the solution has become warmed by the neutralization, a few drops of acid

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<sup>1</sup>This *Journal*, viii, p. 83, 1910

in excess should be added to insure complete solution of the calcium phosphate, the solution cooled and the neutralization repeated. After the solution is just acid to alizarin, 10 cc of  $\frac{3}{4}$  hydrochloric acid and 10 cc of 2.5 per cent oxalic acid are added and the solution is brought to the boiling point and kept gently boiling until the calcium oxalate is coarsely granular. The flask should be kept covered with a watch-glass to prevent spattering. Three per cent ammonium oxalate is then added, a few drops at a time, to the boiling solution, waiting after each addition until the resulting precipitate has become coarsely crystalline. The amount of ammonium oxalate to be added depends on the amount of calcium in solution. Twice the amount necessary to combine with all the calcium is sufficient. After the calcium oxalate has become coarsely crystalline and has settled to the bottom of the flask, it should be frequently stirred up in the liquid to prevent the latter from boiling over suddenly. After the precipitate has become crystalline and has settled, the solution is allowed to cool to room temperature. When cold, 8 cc of 20 per cent sodium acetate solution are added to it slowly and with constant stirring. The solution is then allowed to stand in a cool place from four to eighteen hours, filtered cold and washed free of chlorides with cold 1 per cent ammonium oxalate solution. The precipitate is allowed to dry and then incinerated with the filter paper in a platinum crucible. It is finally heated in a blast lamp to constant weight. Details for the determination of magnesium need not be given here.

The points investigated were the following: (1) The possible substitution of ordinary concentrated hydrochloric acid for  $\frac{3}{4}$  hydrochloric acid, the possible omission (2) of ammonium oxalate, (3) of oxalic acid, (4) of heating, (5) of standing over night, (6) the possible substitution of shaking for either heating or standing over night, (7) the determination of the calcium oxalate by titration with potassium permanganate, (8) the separation and washing of the calcium oxalate by means of the centrifuge instead of by filtration.

Various combinations of these changes were tried. Each change in detail was considered with respect to accuracy, simplicity and rapidity.

#### A GRAVIMETRIC DETERMINATIONS

1 *Use of concentrated hydrochloric acid instead of  $\frac{3}{4}$  hydrochloric acid.* A number of determinations on different samples of acid showed that ten drops of concentrated hydrochloric acid (sp gr, 1.20) from a 5 cc pipette are practically equivalent to 10 cc of  $\frac{3}{4}$  hydrochloric acid. Since good check results were always obtained in a large number of determinations in which concen-

trated hydrochloric acid was used instead of  $\frac{3}{4}$  acid, this modification of the procedure was adopted and used throughout in the analyses reported in this paper

A solution of calcium chloride was prepared and the calcium in 50 cc determined by the standard method already described with the following results <sup>1</sup>

1	2	3	101	102
0 1434	0 1433	0 1436	0 1434	0 1435

The solutions filtered clear and no precipitate formed in the filtrate on standing. This solution was used in the subsequent analyses numbered 4-15 and 100-110

2 *Omission of boiling and omission of oxalic acid* Analyses numbered 100 and 100a, were carried out just as were analyses 1, 2 and 3, except that no oxalic acid was added and the solution was not boiled. Nos 100b and 100c were carried out in the same way except that they were shaken ten minutes after the addition of the sodium acetate instead of standing over night

100	100a	100b	100c
0 1342	0 1303	0 1416	0 1433

These results are very low, partly due to the passage of the very fine precipitate through the filter paper. The next analyses were designed to determine whether the low results were due to the omission of the oxalic acid, of boiling, or of both

3 *Omission of oxalic acid* These analyses were carried out just as were Nos 1, 2 and 3, except that the addition of oxalic acid was omitted and an equivalent amount of ammonium oxalate added instead

4	5	104	104a	105	105a
0 1432	0 1432	0 1433	0 1433	0 1430	0 1434

The results are only slightly low, so that the omission of oxalic acid alone does not account for the low results of analyses Nos 100 and 100a. The precipitate is so fine, however, that it is troublesome to wash it out of the flask and it clogs the filter paper so

<sup>1</sup>Certain observations were checked by independent determinations by a second chemist (Miss Fales). These check analyses are designated by numbers above 100

that the liquid filters very slowly. Sometimes a slight cloudiness is observed in the filtrate, this in time settles out a little and can be seen when the filtrate is shaken. This is probably due to the fraction of a milligram of calcium oxalate which passes through the paper and is responsible for the slightly low results. The fineness of the precipitate can be accounted for by the omission of the oxalic acid. Calcium oxalate is somewhat soluble in an acid solution so that when oxalic acid is added a part only of the calcium oxalate is precipitated. Boiling makes the precipitate still more soluble. It is a well-known fact that if a crystalline precipitate is somewhat soluble, the crystals grow larger—the smaller crystals going into solution, the larger crystals growing by accretion. And so, in the case of the calcium oxalate, digestion in the hot acid solution gives the precipitate an opportunity to form in large crystals, and when the ammonium oxalate and sodium acetate are subsequently added the rest of the calcium oxalate comes down and precipitates partly, at any rate, on the large crystals. The digestion over night brings about further growth of the large crystals at the expense of the fine precipitate.

4 *Omission of boiling* Two analyses were carried out just as were Nos 1, 2 and 3, except that the solution was not heated, precipitation taking place in the cold. The results were

6	7
0 1425	0 1420

Although low, they are not so low as the results of Nos 100 and 100a. The low results of Nos 100 and 100a are therefore not due to omission of either oxalic acid or of boiling alone but to the omission of both. The precipitate is so fine that some may pass through the filter. A filtrate which is apparently practically clear at first may show a fine precipitate on standing over night. Filtration is slow for the fine precipitate soon clogs the paper. The fineness of the precipitate is accounted for by omission of digestion in the hot liquid as explained above. One advantage in the omission of heating was noted, viz the precipitate does not stick to the sides of the flask and hence less water is necessary in transferring it to the filter paper.

5 *Substitution of shaking for standing over night* Four analyses were carried out as were Nos 1, 2 and 3, except that the

flasks were closed with rubber stoppers and shaken for ten minutes after the addition of sodium acetate instead of being allowed to stand over night

9	10	109	110
0 1436	0 1435	0 1434	0 1436

6 *Shaking instead of boiling before addition of sodium acetate*  
When the procedure used in Nos 1, 2 and 3 was modified to the extent of shaking for ten minutes after the addition of ammonium oxalate and again for ten minutes after the addition of sodium acetate, boiling after the addition of both oxalic acid and ammonium oxalate being omitted, it was found that the precipitate was fairly coarsely crystalline, filtration was easy and no precipitate appeared in the filtrate upon standing. Assuming that the results would have been correct, the precipitates were not ignited and weighed.

7 *Omission of boiling, shaking instead of standing over night*  
In analyses which were carried out exactly as were Nos 6 and 7, (*i e*, boiling omitted) except that after the addition of sodium acetate the flasks were closed with rubber stoppers, the solution shaken for ten minutes and filtered immediately instead of after standing over night, a very fine precipitate passed through the paper as in analyses Nos 6 and 7. For this reason the analyses were not finished.

In analyses in which both boiling and standing over night were omitted, the flasks being stoppered and the solutions shaken after the addition of the oxalic acid, after the addition of the ammonium oxalate and again after the addition of the sodium acetate, it was found that the precipitate was coarsely crystalline, filtration was easy and the filtrate remained clear upon standing. Assuming that the results would have been correct, the analyses were not finished. One advantage in shaking as compared with heating is that the precipitate does not adhere to the sides of the flask and hence is easily washed on to the filter. It is a question, however, which is the simpler technique.

The same result was noted in analyses in which boiling and standing over night were omitted, the flasks being shaken for ten minutes twice, *viz* after addition of oxalic acid and after addition of sodium acetate.

14	15
0 1434	0 1435



The results are correct, precipitates coarsely crystalline, filtration easy, the filtrates remaining clear on standing. The precipitate does not adhere to the sides of the flask.

The results<sup>1</sup> thus far outlined indicate that, in carrying out the method, 10 drops of concentrated hydrochloric acid may be used instead of 10 cc of  $\frac{N}{2}$  acid, that the oxalic acid should not be omitted, that boiling may be omitted if the solution is shaken for ten minutes after the addition of the oxalic acid, and that standing over night may be omitted if shaking for ten minutes after the addition of the sodium acetate be substituted.

### B TITRATION WITH POTASSIUM PERMANGANATE

The next experiments were designed to determine whether the calcium could be accurately determined by titration of the oxalate with potassium permanganate. This method is sometimes recommended, but as ordinarily carried out it can not give absolutely accurate results. It has been shown by Richards<sup>2</sup> and myself<sup>3</sup> that if the calcium oxalate precipitate be washed with distilled water, some dissolves, making the results low. If the precipitate be washed with dilute ammonium oxalate solution in the manner described, the ammonium oxalate clinging to the precipitate will react with the permanganate and the result will be high. It seemed probable, however, that if the precipitate were washed free of impurities with dilute ammonium oxalate and then washed a few times with very small amounts of cold distilled water, allowing thorough draining after each washing, these errors could be minimized. Accordingly, three sets of analyses were made as follows:

1 Calcium oxalate was precipitated, filtered and washed with dilute ammonium oxalate in the usual manner. The precipitate was then titrated with potassium permanganate to determine how much too high washing with ammonium oxalate makes the result.

<sup>1</sup>These conclusions are based not only upon results given here, but also upon similar results of a number of other experiments in which calcium oxalate was precipitated in these various ways in preparation for the volumetric determinations. The results given above suffice to bring out the points desired.

<sup>2</sup>*Proc of the Amer Acad of Arts and Sciences*, LVVI, p 277, 1901

<sup>3</sup>*Loc cit*

2 Calcium oxalate, precipitated and filtered as before, was washed with cold distilled water Titration with permanganate indicated the extent of loss caused by the solubility of calcium oxalate in distilled water

3 Calcium oxalate, precipitated and filtered as before, was washed free from chlorides with 0.5 per cent ammonium oxalate solution This was followed by washing three times with cold distilled water, each time using only enough to fill the funnel one-half to two-thirds full and allowing thorough draining each time

For these analyses a new solution of calcium chloride was used Three determinations of the calcium in 50 cc by the standard method yielded the following

<sup>16</sup>	<sup>17</sup>	<sup>18</sup>
0.1500	0.1504	0.1502

The results of the three sets of analyses outlined above were as follows

1 Washing with 0.5 per cent ammonium oxalate

<sup>22</sup>	<sup>23</sup>
0.1545	0.1554

2 Washing with distilled water <sup>1</sup>

<sup>24</sup>	<sup>25</sup>
0.1497	0.1497

3 Washing with ammonium oxalate and then with distilled water

<sup>26</sup>	<sup>27</sup>
0.1502	0.1501

These results show that calcium may be determined by titration of the oxalate with potassium permanganate if washing with dilute ammonium oxalate is followed by washing three or four times with cold distilled water

### C EFFECT OF URIC ACID

In considering the applicability of the method for the estimation of calcium in the urine the possible effect of the presence of uric acid had to be

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<sup>1</sup>It was noted in these and other analyses that the size of the crystals makes as great or greater difference than the nature of the washing fluid If the precipitate is coarsely crystalline, even cold distilled water alone may be used for washing If the precipitate is very finely crystalline, even 0.5 per cent ammonium oxalate carries a little into solution which precipitates out later

*determined* When calcium is precipitated as oxalate in neutral or slightly acid solution and allowed to stand over night, uric acid may be precipitated. Such contamination of the precipitate would not affect the accuracy of the gravimetric method, since the precipitate is ignited before weighing. It would, however, interfere with the volumetric method. The possible prevention of the precipitation of uric acid will be discussed later. On the assumption that it is precipitated, as a means of getting rid of it I have thought of washing the calcium oxalate with sodium hydroxide solution. It was found, however, that even fairly strong sodium hydroxide does not easily dissolve the uric acid contaminating a calcium oxalate precipitate. Furthermore, even dilute sodium hydroxide dissolves some of the calcium oxalate. I have concluded, therefore, that if calcium oxalate, precipitated from urine, is contaminated with uric acid, as indicated by a reddish color of the precipitate, the calcium should not be determined by titration.

#### D USE OF THE CENTRIFUGE

A number of experiments were undertaken to determine whether it might not be of advantage to use the centrifuge in separating and washing the precipitated calcium oxalate instead of filtering in the usual way. They showed that while accurate results may be obtained by its use, the work involved and time consumed is greater than when filtration is employed.

#### E USE OF GOOCH CRUCIBLE OR HARDENED FILTER POINT AND SECTION

When Gooch crucibles were tried, it was found that thin layers of asbestos did not retain the precipitate. Where layers thick enough to retain the precipitate were used, they soon clogged up and prevented passage of fluid.

#### F EFFECT OF PHOSPHATES, MAGNESIUM AND SMALL AMOUNTS OF IRON UPON THE RESULTS

In order to convince myself that the presence of magnesium, phosphates or iron would not affect the accuracy of the calcium determination if boiling and standing over night were omitted, the following analyses were made. Nos. 70 and 71 were carried out on a pure calcium chloride solution, Nos. 72 and 73 on the same solution to which were added equivalent quantities of magnesium chloride and sodium phosphate, together with a little ferric ammonium sulphate.

70	71	72	73
0.1377	0.1377	0.1376	0.1377

### PART II THE DETERMINATION OF CALCIUM IN URINE

The method which has been described in Part I, when applied to the estimation of calcium in urine, gave accurate results. Certain steps in it were, however, found to be unnecessary, even inad-

visible, owing partly to the small amounts of calcium, partly to the character of the urine

Fresh urine from a number of subjects was mixed and the calcium content in 200 cc estimated by the method described last year The results follow

<sup>1</sup>	<sup>2</sup>
0 0242	0 0242

1 *Omission of ammonium oxalate* Two analyses in which the addition of ammonium oxalate was omitted showed that this reagent was superfluous in the estimation of calcium in urine

<sup>3</sup>	<sup>4</sup>
0 0243	0 0242

2 *Shaking instead of standing over night* It has been shown in Part I that shaking of the solution may be substituted for standing over night when there is considerable calcium present The fine precipitate which comes down on shaking after the addition of sodium acetate has in that case a large amount of coarsely granular precipitate to which to attach itself In the case of urine the amount of precipitate that comes down before adding sodium acetate is small Hence shaking for ten minutes is insufficient to give a coarsely granular precipitate In two analyses, in which this modification was introduced, the calcium oxalate was so finely divided that some passed through the filter The analyses therefore were not finished

3 *Effect of boiling the solution* In all these analyses filtration was slow and washing of the precipitate difficult because the filter became clogged When boiling was omitted this was not the case Boiling seems to precipitate a very small quantity of sticky, slimy substance, possibly mucus, which coats the calcium oxalate precipitate and the filter paper, impeding filtration Boiling also seemed to prevent the formation of a coarse precipitate, for when the urine was not boiled the precipitate was coarse enough to be retained by the filter

It was noted, further, that, if the urine is boiled and then allowed to stand as usual over night, the calcium oxalate precipitate is occasionally contaminated with uric acid In no case did this occur when boiling was omitted While it is difficult to assign

a reason for this, the point is of interest, for the calcium can be determined by titration with permanganate if it can be precipitated free from uric acid

1 *Shaking instead of boiling* Analyses carried out as directed in my previous paper, except that, instead of boiling the urine, it was vigorously shaken in a stoppered flask, gave good results. Filtration was easy and the filtrate remained clear

9	10
0 0242	0 0241

5 *Shaking instead of boiling omission of ammonium oxalate* In the analyses, the results of which follow, vigorous shaking was substituted for boiling and the addition of ammonium oxalate was omitted. The results are good, filtration was easy and the filtrate remained clear

13	14
0 0242	0 0241

6 *Omission of boiling and of ammonium oxalate shaking instead of standing over night* In the analyses following, a mixed urine was used which gave 0 0275 gram of calcium oxide in 200 cc by the standard method. It was found that shaking could not be substituted for standing over night if the urine was boiled, because the fine precipitate so obtained passed through the filter. This was not the case when the urine was not boiled as the results of analyses 15 and 16 show

15	16
0 0274	0 0275

Filtration was easy and the filtrate remained clear on standing

7 *Urine not boiled or shaken* In the case of pure solutions, in which calcium is fairly concentrated, it has been shown that either boiling or shaking is essential. Neither is essential in the case of urine as is shown by the results of analyses 17-20 in which both boiling and shaking were omitted. In 17 and 18 ammonium oxalate was added, not in 19 and 20. In all, the urine was allowed to stand over night, after the addition of sodium acetate

17	18	19	20
0 0275	0 0275	0 0275	0 0274

Filtration was more rapid in those analyses in which ammonium oxalate was omitted

In the analysis of urine, as in the case of pure solutions, the use of hardened filter points or of Gooch crucibles and suction was found to be impracticable. Concerning titration with permanganate, the considerations previously outlined are applicable. It is advantageous to use filtered urine for the analysis, and in urines which contain small amounts of calcium, litmus paper should be used in adjusting the reaction in the first step of the analysis.

#### SUMMARY

I For the determination of calcium in pure solutions, which may contain also magnesium, phosphates and small amounts of iron, or in the ash of food or feces, the best procedures as developed in this work are as follows

**SOLUTIONS REQUIRED** 2.5 per cent oxalic acid, 3 per cent ammonium oxalate, 20 per cent sodium acetate

1 The solution is brought to a volume of 75 to 150 cc. Concentrated ammonia water is added drop by drop till the solution is just alkaline, as shown either by the appearance of a precipitate of calcium and magnesium phosphates or by the use of an indicator (alizarin red or litmus paper)

2 Concentrated hydrochloric acid is added drop by drop until the solution is just acid, as shown either by the disappearance of the precipitate or by an indicator. In the presence of iron an indicator must be used.

3 Ten drops of concentrated hydrochloric acid (sp. gr., 1.20), approximately equivalent to 10 cc. of  $\frac{7}{8}$  HCl, are added.

4 Ten cubic centimeters of 2.5 per cent oxalic acid are added.

5 Either of two procedures may here be used.

a The solution is boiled until the precipitated calcium oxalate is coarsely crystalline,<sup>1</sup> and then an excess of 3 per cent ammonium oxalate is slowly added to the boiling solution and the boiling continued until the precipitate is coarsely crystalline.<sup>2</sup>

b The flask, closed with a rubber stopper, is shaken vigorously for ten minutes. An excess of 3 per cent ammonium oxalate is then added.

<sup>1</sup> If but little calcium is present, nothing precipitates at this point.

If but little calcium is present, it is not necessary to add oxalate.

6 The solution is cooled to room temperature and 8 cc of 20 per cent sodium acetate added (In case of ash of feces, add 15 cc)

7 The solution may either be (a) allowed to stand over night or (b) stoppered and vigorously shaken for ten minutes

8 The calcium oxalate is filtered off on a small ash-free paper and washed free from chlorides with 0.5 per cent ammonium oxalate solution

9 Either of two procedures may next be followed

a The precipitate and filter are dried, burned in a platinum crucible to calcium oxide and brought to constant weight by heating in a blast-lamp

b The precipitate is washed three times with cold distilled water, filling the filter about two-thirds full and allowing it to drain completely before adding more. A hole is made in the paper and the calcium oxalate washed into the flask. The volume of the fluid is brought up to about 50 cc and 10 cc of concentrated sulphuric acid are added. The oxalate is titrated immediately with standard potassium permanganate

Determination of magnesium in the filtrate may be carried out as described in my previous paper. Or the filtrate may be evaporated to dryness in a porcelain dish after the addition of nitric acid and the residue heated over a free flame until the ammonium salts are destroyed and the residue fuses. After cooling, the residue is taken up in water and a little hydrochloric acid, and the magnesium precipitated as usual. Another simple method of destroying the organic matter is to add 3 to 5 cc of concentrated sulphuric acid to the filtrate and evaporate in a small Kjeldahl flask until the water is removed. The sulphuric acid may then be neutralized with ammonium carbonate and the magnesium precipitated as usual.

In the analysis of ash of feces, carried out by the usual method the calcium oxalate is apt to precipitate in a very finely divided condition. Filtration is slow and loss may result on this account. This difficulty may be obviated as has been indicated, by the addition of 15 cc instead of 8 cc of sodium acetate. I have shown that, if the solution is kept cool, no phosphate or magnesium is brought down by this excess of sodium acetate.

II For the determination of calcium in urine, the following method is recommended

SOLUTIONS NEEDED 2.5 per cent oxalic acid, 20 per cent sodium acetate

- 1 If the urine is alkaline it is made neutral or slightly acid
- 2 The neutral or slightly acid urine is filtered
- 3 Two hundred cubic centimeters of urine are used for analysis. If only faintly acid to litmus paper, 10 drops of concentrated hydrochloric acid are added

If the urine is strongly acid, it may be made just alkaline with ammonia and then just acid with hydrochloric acid. If the urine is turbid or contains little calcium, litmus paper should be used in determining these changes, otherwise, the appearance and disappearance of a precipitate of phosphates are sufficiently accurate indicators. Ten drops of concentrated hydrochloric acid (sp. gr. 1.20) are then added.

- 4 Ten cubic centimeters of 2.5 per cent oxalic acid are next added

- 5 Eight cubic centimeters of 20 per cent sodium acetate are added

- 6 The urine is either allowed to stand over night at room temperature or is shaken vigorously for ten minutes

- 7 The calcium oxalate is filtered, and washed free from chlorides with 0.5 per cent ammonium oxalate solution

- 8 *a* The precipitate may then be dried, ignited, heated in a blast-lamp to constant weight and weighed as calcium oxide

- b* If free from uric acid, the calcium oxalate may be washed three times with distilled water and estimated by titration with permanganate as described in the outline of the method for pure solutions

Magnesium is determined in the filtrate just as in the case of pure solutions





# A STUDY OF THE OPTICAL FORMS OF LACTIC ACID PRODUCED BY PURE CULTURES OF BACILLUS BULGARICUS<sup>1</sup>

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(Received for publication, August 13, 1911)

In 1906 Grigoroff<sup>2</sup> isolated a bacillus from yoghurt, the Bulgarian fermented milk, which he named *B. bulgaricus*. This name has since been used to include a general group of bacteria, especially characterized by their ability to produce a higher percentage of acid than other lactic acid forming bacteria. This group of bacteria attracted special attention after Metchnikoff<sup>3</sup> suggested that it be established in the alimentary tract to prevent the growth of harmful organisms. The investigations of Freudenreich<sup>4</sup> and Hastings<sup>5</sup> have shown it to be of great economic importance in the dairy industry.

Numerous observations have been made on the characteristics of bacteria isolated from a single source, which apparently belong to this group, but few studies have been made of the group as a whole, with a view of correlating the various strains. Such an investigation, however, was made by Heineman and Hefferan<sup>6</sup>. One of their conclusions was that the lactic acid produced was

<sup>1</sup>Submitted to the faculty of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>2</sup>Grigoroff *Deutsche med. Wochenschr.*, **xxi**, p. 73, 1895.

<sup>3</sup>Metchnikoff *Prolongation of Life*, p. 161, 1903.

<sup>4</sup>Freudenreich *Landwirtschaftliches Jahrbuch der Schweiz*, **xviii**, p. 525, 1904.

<sup>5</sup>Hastings *Science*, **xxviii**, p. 656, 1903. Hastings and Hammer *Research Bulletin No. 6*, University of Wisconsin Agricultural Experiment Station.

<sup>6</sup>Heineman and Hefferan *Journal of Infectious Diseases*, **vi**, p. 304, 1909.

always inactive Bertrand and Weisweiler<sup>1</sup> made a chemical study of a culture of *B. bulgaricus* obtained from Metchnikoff and found that the lactic acid produced was a mixture of the inactive and dextro forms. The lactic acid obtained from Cheddar cheese by Suzuki, Hastings and Hart<sup>2</sup> was in some cases inactive and in others a mixture of inactive and dextro. From these observations we concluded that there were probably different strains of *B. bulgaricus* which could be differentiated by the optical form of the lactic acid produced. This belief, together with the hope of contributing something to the important problems of the relation of stereochemistry to biological processes, led us to undertake this investigation. This paper is devoted to a discussion of the results obtained and some of the literature bearing directly on this subject.

Certain points in the history of lactic acid are of particular interest in connection with this study. Lactic acid was discovered in sour milk by Scheele about 1780. Liebig<sup>3</sup> isolated an acid from muscle extract in 1847 which he thought was identical with the acid of sour milk. But the following year Englehart<sup>4</sup> showed that the zinc and calcium salts of the two acids differed in their solubility, water of hydration and point of decomposition. He concluded that the acids were not identical but isomeric. The explanation of the isomerism of these two acids was a problem which received the attention of nearly every prominent organic chemist of the time. In a paper published in 1873 Wislicenus,<sup>5</sup> who had previously shown that, of the two acids, only sarco or muscle lactic acid was optically active, summed up the evidence in favor of their structural identity yet dissimilar properties, and concluded that this could only be explained by assuming a different arrangement of the atoms in space. He proposed to call this type of isomerism, geometrical isomerism. The influence of these speculations on the subsequent development of theoretical chemistry is apparent from the statement of van't Hoff<sup>6</sup> that the reflections which led him to his theory of the asymmetric

<sup>1</sup>Bertrand and Weisweiler *Annales de l'Institut Pasteur*, **xx**, p. 977, 1906

<sup>2</sup>Suzuki, Hastings and Hart *This Journal*, **vii**, p. 431, 1910

<sup>3</sup>Liebig *Liebig's Annalen*, **lxii**, p. 326, 1847

<sup>4</sup>Englehart *Ibid.*, **lxv**, p. 359, 1848

<sup>5</sup>Wislicenus *Ibid.*, **clxvii**, p. 302, 1873

<sup>6</sup>van't Hoff *Introduction to Chemistry in Space*

carbon atom were suggested by the ideas of Wislicenus on the isomerism of sarco and fermentation lactic acids. This problem was finally solved by a study of biochemical processes. In 1889 Nencki and Sieber<sup>1</sup> isolated a micrococcus which in pure cultures produced only dextro lactic acid. The following year Schardinger<sup>2</sup> isolated a bacillus which produced only laevo lactic acid. He mixed equal quantities of zinc lactates prepared from sarco-lactic and from the laevo acid produced by his organism, and found that inactive zinc lactate separated out. This established beyond question the relation of the active and inactive lactic acids. The resolution by chemical means of the inactive fermentation acid into its active components has been accomplished by Purdie and Walker<sup>3</sup>.

It is evident from what has preceded that fermentation lactic acid is not, as is frequently stated in text-books, always inactive, but may also be either dextro or laevo rotatory, according to the type of organism employed in the fermentation. Numerous attempts have been made to formulate a definite relation between the ferment and the form of acid produced. Nencki<sup>4</sup> suggested, after isolating his dextro acid forming coccus, that an organism always produced the same optical form of acid, and that this would serve as a certain means of identification. Pére<sup>5</sup>, Kayser<sup>6</sup> and Pottevin<sup>7</sup> have obtained results which indicate that the same organism, according to the conditions of growth, can produce either dextro, laevo or inactive acid. The results of these French investigators have been called in question by Kozar<sup>8</sup> in an investigation to explain the paradoxical observation of Gunther and Thierfelder<sup>9</sup> that, while the acid of spontaneously soured milk was usually inactive, *B. lactis acidus*, the chief factor in the souring of milk, when in pure cultures produced only the dextro form

<sup>1</sup> Nencki and Sieber *Monatshefte für Chemie*, **v**, p. 532, 1889

<sup>2</sup> Schardinger *Monatshefte für Chemie*, **xi**, p. 545, 1890

<sup>3</sup> Purdie and Walker *Transactions of the Chemical Society*, 1892, **ix**, p. 754, 1892

<sup>4</sup> Nencki *Centralblatt für Bacteriologie*, **ix**, p. 304, 1891

<sup>5</sup> Pére *Annales de l'Institut Pasteur*, **vii**, p. 737, 1893, **viii**, p. 63, 1898

<sup>6</sup> Kayser *Ibid*, **ix**, p. 737, 1895

<sup>7</sup> Pottevin *Ibid*, **xii**, p. 49, 1898

<sup>8</sup> Kozar *Zeitschrift für Hygiene*, **xxi**, p. 337, 1899

<sup>9</sup> Gunther and Thierfelder *Archiv für Hygiene*, **xxv**, p. 164, 1895

solution to release the lactic acid from its calcium and casein combinations, and the lactic acid extracted with ether in a Kutscher-Steudel continuous extractor for at least seventy-two hours. If the solution did not then give Uffelmann's test for lactic acid, the extraction was considered complete. After evaporating off the ether the lactic acid was dissolved in water, a slight excess of zinc carbonate added and the solution boiled for several minutes. The excess of zinc carbonate was then filtered off and the residue washed with hot water. The filtrate and washings were evaporated to crystallization at 60°–70° C. When crystals appeared, the solution was allowed to evaporate at room temperature until only a few cc of mother liquor remained. This was then set aside in a cool place and after a few hours the mother liquor was drained off and the crystals washed with a small volume of cold water. The mother liquor and washings were again evaporated for a second crop of crystals. The zinc lactate recovered was combined and a portion of the air-dried salt dehydrated at 106°C. Englehart<sup>1</sup> showed that the zinc salt of the inactive acid crystallized with three molecules of water, while the corresponding salt of the active acid carries but two molecules of water. Owing to the small specific rotation ( $\approx 3^\circ$ ) of the active acid a determination of the water of hydration of the zinc salt has been generally regarded as the most satisfactory method for distinguishing these acids. When the zinc lactate is pure it dehydrates readily to a constant weight in from one to two hours, but when impure it loses the last traces of the water slowly and decomposes so readily that the point of complete dehydration is not sharply marked. One part of the zinc salt of the inactive acid is soluble in 58 parts of water at 16° C and crystallizes out quite pure. At the same temperature one part of the zinc salt of either active acid is soluble in seventeen parts of water and generally carries enough impurities to make it dehydrate with difficulty. In such cases satisfactory results were obtained by dehydrating 0.4 to 0.5 gram samples for fifteen minute periods until the loss in weight did not exceed 0.3 mg. The zinc oxide in the dehydrated salt was determined in nearly every case as a check on the accuracy of the dehydration and the purity of the zinc lactate. A satur-

<sup>1</sup>Englehart *loc cit*

ated solution of the zinc lactate was always examined in the polariscope before crystallization. In case this preliminary examination and also the water of hydration showed that the salt was probably a pure active zinc lactate, the specific rotation was determined at 22° C in a 4 dm tube and at a concentration of 4.122 grams of anhydrous zinc lactate per 100 cc. The specific rotation of zinc lactate varies with the concentration. At a concentration comparable to that used in these determinations Hoppe-Seyler and Araki<sup>1</sup> give 7.552°. The rotation of the zinc lactate is opposite in sign from that of the free acid. In order to get comparative values of the percentage of acid produced, 100 cc of sterile milk were inoculated with the pure culture and, after thirty days incubation at 38°, the acidity to phenolphthalein was determined by titration against  $\frac{1}{10}$  sodium hydrate, and the acidity expressed in per cent of lactic acid.

#### DISCUSSION OF DATA

The cultures from human saliva designated I, II and III were obtained by inoculating sterile milk from three separate colonies on the agar plate. The optical form of the acid and also the per cent of acidity reached in thirty days indicate that the cultures were all of the same bacillus. In order to get some data on the form of acid produced under varying cultural conditions, number II was grown in a medium containing 0.5 per cent of peptone and 2 per cent of lactose and also on a medium containing 0.5 per cent of peptone and 2 per cent of glucose. Only a small amount of acid was recovered from these cultures, but in both instances it was of the same optical form as that produced in sterile milk. From these results we must conclude that, so far as this particular organism is concerned, varying both the source of nitrogen and of carbohydrate does not affect the optical form of the acid produced. The ether extract from these cultures gave only a trace of volatile acids.

The morphology and cultural characteristics of the bacillus isolated from human feces were identical with those of the bacillus from saliva. This organism, like the one from saliva, produced

<sup>1</sup>Hoppe-Seyler and Araki. *Zeitschrift für physiologische Chemie*, x, p. 371, 1895.

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*Data*

SOURCE OF ORGANISM	HYDRATE WATER IN Zn LACTATE	ZINC OXIDE IN DEHY- DRATED SALT	$[\alpha]_D^{22}$ OF Zn LAC- TATE 4 122 GRAMS	OPTICAL FORM OF ACID	ACIDITY IN THIRTY DAYS
	<i>per cent</i>	<i>per cent</i>	<i>per 100 cc</i>		<i>per cent</i>
Theory for active acid	12 89	33 33	$\pm 7.522$		
Theory for inactive acid	18 18	33 33	$\pm 0.0$		
Human saliva, I	12 95	33 38	-7 28	Dextro	2 68
Human saliva, II	12 84	33 27	-7 16	Dextro	2 45
Human saliva, III	13 02	33 33	-7 16	Dextro	2 52
Human saliva, II (peptone- lactose media)	13 09	33 14		Dextro	
Human saliva, II (peptone- glucose media)	12 83	33 22		Dextro	
Human feces	13 02	33 26	-7 28	Dextro	2 56
Duplicate culture	13 13	33 20	-7 28	Dextro	
Brewers' malt	12 38	33 44	-7 35	Dextro	1 40
Duplicate culture (after steam distillation)	12 95	33 41	-7 46	Dextro	
Kraut	12 67	34 51		Dextro	1 47
Duplicate culture (after steam distillation)	13 21	32 91	-6 97	Dextro	
Horse feces	18 19	33 38	$\pm 0.0$	Inactive	0 58
Cow feces	17 94	37 14	$\pm 0.0$	Inactive	1 20
Duplicate culture (after steam distillation)	18 12	33 43	$\pm 0.0$	Inactive	
Milk soured at 38° C, I	16 39	35 38	+	<i>i</i> and <i>l</i>	
Milk soured at 38° C, II	18 01	33 43	+	<i>i</i> and <i>l</i>	
Cheddar cheese I	12 71	33 33	-7 46	Dextro	
Cheddar cheese II	13 27		-7 46	Dextro	2 12
Cheddar cheese III	14 11	33 53		<i>d</i> and <i>i</i>	2 02
Cheddar cheese IV	14 78	33 66		<i>d</i> and <i>i</i>	
Cheddar cheese V	18 10	33 51	$\pm 0.0$	Inactive	1 40
Cheddar cheese VI	17 94	33 66	$\pm 0.0$	Inactive	
Cheddar cheese VII	17 91	33 55	$\pm 0.0$	Inactive	
Cheddar cheese VIII	17 99	33 87	$\pm 0.0$	Inactive	
Cheddar cheese IX	17 48	33 22	+	<i>i</i> and <i>l</i>	
Cheddar cheese X	17 24	33 27	+	<i>i</i> and <i>l</i>	
Cheddar cheese XI	12 98	33 52	+7 46	Laevo	

pure dextro lactic acid and also attained very nearly the same acidity in 30 days. These results show the presence of identical, powerful acid-producing strains of *B. bulgaricus* in human saliva and feces.

The bacilli from kraut and malt were very similar to each other. The per cent of acidity attained was less than that of the cultures from saliva and feces, but the form of the lactic acid produced was again in both cases pure dextro. Both cultures contained volatile acids approximating 4 per cent of the total acidity. The considerable volatility of lactic acid with steam does not permit of an accurate determination of small amounts of volatile acids in its presence. The culture from malt had an appreciable odor of butyric acid. The presence of volatile acids was further shown by analyses of the zinc lactate prepared from the ether extract before and after distillation with steam. The latter conformed more nearly to the theoretical values than the former.

The organism isolated from horse feces possessed the morphology and cultural characteristics usually ascribed to *B. bulgaricus* but reached an acidity of only 0.58 per cent in thirty days. This fact might be interpreted to mean that it should not properly be classed as *B. bulgaricus*. Unlike the cultures previously studied it produced pure inactive lactic acid. It possessed a high optimum temperature, did not coagulate milk at all at room temperature and at 38° only after a period of two weeks or longer. The acidity increased uniformly throughout the period of incubation. These are all characteristics of *B. bulgaricus*.

The first culture of the bacillus from cow feces studied gave a zinc lactate containing 17.94 per cent of water and 37.14 per cent of zinc oxide. The figure for zinc oxide indicated the presence of acids other than lactic. A duplicate culture was distilled with steam before neutralizing with zinc carbonate. The volatile acids calculated to lactic amounted to 20 per cent of the total acidity. The zinc lactate recovered was inactive. Although this was a large, irregularly staining bacillus and produced an acidity of 1.20 per cent it is doubtful whether it belonged to the *B. bulgaricus* group because of the large amount of volatile acids produced.

To obtain a culture from milk, a small bottle was completely filled with milk, tightly stoppered and incubated until a stained smear showed the presence of long slender bacilli. The milk was



then plated out on whey agar. The pure culture contained a mixture of inactive and laevo acids. Analysis of the dehydrated zinc lactate showed that it was not pure. The zinc lactate was decomposed with hydrogen sulphide and the zinc sulphide filtered off. On distilling with steam only a trace of volatile acids was obtained. The solution was then examined for succinic acid. To separate succinic and lactic acids advantage was taken of the insolubility of barium succinate in 60 per cent alcohol. The white precipitate thrown down by the alcohol was filtered off, decomposed by the cautious addition of dilute sulphuric acid, and the filtrate from the barium sulphate evaporated to dryness. When the residue was heated copious white fumes were given off which had the characteristic choking effect of succinic anhydride. The white sublimate obtained after crystallization from water melted at  $178^{\circ}$ – $179^{\circ}$  C. The literature gives  $180^{\circ}$ – $182^{\circ}$  C for the melting point of succinic acid. We think this established the presence of small amounts of succinic acid in this culture. Another culture isolated from milk in the same manner gave similar results with the exception that the proportion of laevo to inactive lactic acid present was smaller. These cultures died very quickly and before any results were obtained on the per cent of acidity produced. The biological functions of this bacillus appear quite similar to those of Kozai's<sup>1</sup> *B. acidilactici halensis*, which he concluded produced laevo lactic acid and traces of succinic acid. Bertrand and Weisweiler<sup>2</sup> state that small amounts of succinic acid were produced by the culture of *B. bulgaricus* obtained from Metchnikoff.

The cultures isolated from Cheddar cheese were kindly furnished by Miss Evans, assistant bacteriologist in the dairy division of the Bureau of Animal Industry, who is working in cooperation with the Department of Agricultural Bacteriology of the University of Wisconsin. No very definite conclusions can be drawn from these results. It appears, however, that at least four different strains of high-acid organisms were present in these cultures, one which produced pure or nearly pure dextro lactic acid, one which produced inactive acid, one which produced a mixture

<sup>1</sup> Kozai *Zeitschrift für Hygiene*, xxxviii, p. 386, 1901

<sup>2</sup> Bertrand and Weisweiler *loc. cit.*

of laevo and inactive acids and one which produced pure laevo acid. It will be remembered that, with the exception of the last named type, organisms of corresponding acid producing functions were isolated from other sources. We were unable to isolate with certainty succinic acid from any of these cultures.

#### SUMMARY AND CONCLUSIONS

1 Cultures of *B. bulgaricus* were isolated from human saliva, human feces, malt, kraut and Cheddar cheese which produced only dextro lactic acid, from Cheddar cheese which produced dextro lactic acid with a small admixture of inactive acid, from horse feces, cow feces and Cheddar cheese which produced only inactive acid, from milk soured at 38° C and from Cheddar cheese, which produced a mixture of laevo and inactive acids, and from Cheddar cheese which produced pure laevo lactic acid.

2 Among the bacteria which have been included in the *B. bulgaricus* group, there are strains which may be differentiated by the optical form of the lactic acid produced.

3 Varying both the source of nitrogen and of carbohydrate in the culture media did not alter the optical form of the acid produced by the bacillus isolated from saliva.

4 The dextro lactic acid forming strain predominates and has the power to produce a higher percentage of acid than the other strains.

5 Pure cultures of some bacteria may produce inactive lactic acid, a fact which probably necessitates the presence of both dextro and laevo acid producing enzymes in a single organism.

6 The bacilli of the *B. bulgaricus* type in human feces and human saliva are identical.

7 Some strains of *B. bulgaricus* produce small amounts of succinic acid. This may account for the presence of this acid in Cheddar cheese, although we could not definitely isolate it from any cultures obtained from cheese.

I wish to express my indebtedness to Professors E. B. Hart and E. G. Hastings for their advice throughout this work.



# EXPERIMENTAL STUDIES ON CREATINE AND CREATININE<sup>1</sup>

## I THE RÔLE OF THE CARBOHYDRATES IN CREATINE-CREATININE METABOLISM

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(Received for publication, August 14, 1911)

### INTRODUCTORY

Despite the enormous amount of data that have been accumulated in recent years in regard to the occurrence and excretion of creatine and creatinine, our knowledge of the significance of these substances is still far from adequate. The incidence of the excretion of creatinine (cf Mendel, '09 and Myers, '10), is perhaps better understood than that of creatine. Folin ('05a, '05b, '05c), van Hoogenhuyze and Verploegh ('05), Klercker ('06), Closson ('06), and Shaffer ('08b) have shown that the daily output of creatinine, on a meat-free diet, is remarkably constant for the same individual, and is independent of the total nitrogen and volume of the urine. This constancy in the excretion of creatinine indicates that it is of endogenous origin, and as Folin ('05b) says, is an "index of a certain kind of protein-metabolism occurring daily in any given individual." Folin ('06) further believes that the creatinine of the urine has no connection with the muscle creatine, as the latter is converted into creatinine only with great difficulty.

<sup>1</sup> A preliminary report of these studies was presented to the Society for Experimental Biology and Medicine, May 17, 1911

The excretion of creatinine in disease frequently undergoes marked variation<sup>1</sup> Mellanby ('07) found the creatinine-coefficient to be very low in diseases of the liver This was particularly true of individuals with hepatic carcinoma The urines of such patients contained large amounts of creatine Mellanby ('08) believes that the liver is intimately connected with the production and excretion of creatinine He suggests that the liver is continuously forming it from substances brought from other organs by the blood In the developing muscle, this creatinine is transformed into creatine, and stored until the muscles have reached a certain saturation point After this point has been reached, creatinine is continuously excreted

Both creatine and creatinine are excreted in the urine of dogs with Eck fistulas, according to Salaskin and Zaleski ('00), London and Boljarski ('09), and Foster and Fisher ('11) London and Boljarski found that the administration of creatinine to such animals did not increase urinary creatinine Results contradictory to these were obtained by Foster and Fisher, who report that the ingestion of creatinine increases the creatinine output in the urine The results of both investigations are in accord in finding that the feeding of creatine caused no increase in creatine excretion, but was followed by a slight rise in eliminated creatinine

The observations of van Hoogenhuyze and Verploegh ('05) and Shaffer ('08a) as well as of certain earlier investigators, indicate that increased or decreased muscular activity, with adequate food, has *per se* no effect on the excretion of creatinine

Creatine is never normally present in the urine of adult mammals, unless creatine is taken in with the food As early, however, as 1868 Meissner ('68) found that creatine almost entirely replaces creatinine in the urine of birds These observations were subsequently verified by Paton ('09-'10) and Voegtlin and Towles ('11)

In mammals, creatine is excreted in the urine during inanition This was first observed by Benedict ('07), and later verified in

<sup>1</sup>For the literature on the excretion of creatinine in various pathological conditions, see Leathes ('06-'07), Spriggs ('07a, '07b), Benedict and Myers ('07a), Forschbach ('08), Shaffer ('08b), and Levene and Kristeller ('09)

collaboration with Diefendorf (Benedict and Diefendorf, '07) on a fasting woman. At about the same time, but independently, Cathcart ('07a, '07b) noted the excretion of creatine in starvation. Recently this author (Cathcart, '09) has published the results of experiments made upon himself and others in which he reports that creatine is found in the urine, in relatively large amounts, after fasting periods of forty hours. In starvation experiments made upon rabbits, Dorner ('07) noted the elimination of creatine. Similar results were obtained in dogs by Underhill and Kleiner ('08), Richards and Wallace ('08), and Howe and Hawk ('11).

Pathologically, creatine occurs in a variety of conditions. Benedict and Myers ('07b) found it in the urines of a large number of insane patients, most of whom were in poor nutritive condition, which probably accounted for its appearance. In convalescence after typhoid fever Foster ('10) found an elimination of creatine. Shaffer ('08b) observed creatine to be invariably excreted where there was a rapid loss of muscle protein, such as in acute fevers, in the acute stages of exophthalmic goitre, in tumor cachexia, and in women during the first week *post partum*, when the resolution of the muscular wall of the uterus is proceeding most rapidly. This has also been observed in dogs by Murlin ('08-'09). According to this author, the creatine first appeared in the urine two days before parturition, and reached a maximum on the fifth day after parturition. He suggests that the latter date probably marks the maximum of the involution process. Levene and Kristeller ('09) noted the excretion of creatine in a variety of diseases. The largest amounts were found in the urine of patients with anterior poliomyelitis and muscular dystrophy.

In many of these instances—notably fevers and hepatic carcinoma—under-nutrition is undoubtedly an important contributing factor in the production of creatine. In this connection the recently published paper of Underhill and Rand ('10) is of particular interest. These authors found large amounts of creatine in the urines of women with pernicious vomiting of pregnancy, and Underhill suggests that the changes observed in the urine are induced by the accompanying inanition. Evidence tending to substantiate this view is furnished by the observation that the perverted creatine metabolism, as well as the

metabolism of the other nitrogenous constituents of the urine, tends rapidly to resume the normal on the rectal administration of dextrose, without necessarily exerting any influence on the pathological state of the patient. The presence of carbohydrates appears in these experiments to be the all-important factor in preventing the abnormal partition of urinary nitrogen associated with starvation. In the absence of sufficient carbohydrates the energy-yielding substances in the body seem to be utilized with great difficulty. The inability to oxidize carbohydrates may, therefore, explain the observations of Shaffer ('08b) and Dreiholz ('08), and more recently of Krause ('10), Krause and Cramer ('10), and Taylor ('10, '11), that creatine is a constant product of the metabolism of patients with diabetes mellitus.

In consideration, therefore, of such observations as the above, a series of experiments was conducted on starving rabbits, to determine the influence of carbohydrates on the creatine metabolism during a period of inanition unaccompanied by any other abnormal factor.

From two experiments upon geese, Paton ('09-'10) reached the conclusion that the administration of glucose in fasting has no specific action on the excretion of creatine. But his experiments were entirely too few in number, and extended over too short periods of time, to render his results conclusive.

While the present work was in progress, an interesting paper appeared by Cathcart ('09), in which he reports that the creatine excretion induced by fasting is reduced to *nil* by administering a carbohydrate diet "practically nitrogen and fat free," whereas with a fat diet, the amount of creatine excreted is increased. He further states that the addition of protein food (carbohydrate-free) during the fat period, does not markedly reduce the creatine excretion. The experiments were made upon men, which necessarily limited the duration of the fasting to short periods (usually forty hours). The author reports that this inanition always brought about an output of some hundred and fifty milligrams of creatine, which seems surprisingly large for a fast of so short duration. Usually, several days of starvation are necessary to induce the excretion of appreciable amounts of creatine in man (cf. Benedict and Diefendorf, '07, and Underhill and Rand, '10).

The chief criticism, however, of Cathcart's experiments involves the nature of the carbohydrate diet used to reduce the creatine output. This consisted of tapioca, sugar, honey, corn-flour, and banana meal, all of which—with the exception of the sugar—contain small amounts of nitrogen. In one experimental period

TABLE I  
*Cathcart's carbohydrate diet*

ARTICLE OF FOOD	AMOUNT INGESTED PER DAY	N	N INTAKE
	grams	per cent	grams
Banana meal	454	0.64	2.80
Honey	230	0.23	0.53
Total N intake per day			3.33

(Cathcart, '09, p. 316) the diet consisted of banana meal and honey, and contained according to Cathcart's own analyses, 3.33 grams of nitrogen (see Table I). It is true that this is a small nitrogen intake, and that for most purposes the diet might be considered "practically nitrogen free," but, *a priori*, we have no way of knowing whether the nitrogen accompanied by the carbohydrates, or the carbohydrates *per se* are responsible for the reduction in creatine elimination. Chittenden<sup>1</sup> has shown that man can live in perfect health, and remain in nitrogen equilibrium, on an intake of 5 or 6 grams of nitrogen per day, when sufficient carbohydrates and fats are ingested. May it not, therefore, be possible, that an intake of half of the amount of nitrogen necessary to meet the needs of the body is capable, when accompanied by carbohydrates, of exerting some influence on creatine elimination during starvation? Certainly we have no answer to this question in the results of Cathcart. Hence, it was determined to test the influence of a carbohydrate diet *absolutely nitrogen-free* on the creatine elimination in animals during inanition.

<sup>1</sup>Chittenden *Physiological Economy in Nutrition*, New York, 1904.



EXPERIMENTAL PART <sup>1</sup>*Methods*

The animals used in the investigation were large rabbits, previously well fed on oats, cracked corn and carrots. In several experiments the urines were analyzed for three or four days before the fasting periods were begun, in order to determine whether or not creatine is normally excreted by rabbits on a mixed diet. The urine was always collected at the end of twenty-four hour periods, unless otherwise stated, the complete excretion for the day's cycle being obtained by squeezing out the bladder. Total nitrogen was estimated by the Kjeldahl-Gunning method, ammonia nitrogen<sup>2</sup> and preformed creatinine<sup>3</sup> by the Folin methods, and "total creatinine,"<sup>4</sup> *i e*, after conversion of creatine present to creatinine, by the Benedict-Myers modification of the Folin method.

In describing the creatinine determination in rabbits' urine, Dorner ('07) alludes to the appearance of a flocky precipitate after treating with picric acid and sodium hydroxide, the precipitate persisting even after dilution of the mixture to 500 cc. He suggests filtering the solution before making the colorimetric readings. It has been the experience of the writer that this procedure is entirely unnecessary. The precipitate which is composed largely or entirely of phosphates, is so small in amount that it offers no hindrance to matching the color accurately with the standard bichromate solution. Frequently, in sufficiently dilute urines, no visible precipitate appears at all.

Often a difference of three or four minutes in the time the urine is allowed to stand after the addition of the picric acid and sodium hydroxide, produces a variation of a millimeter or more in the colorimetric reading. The maximum depth of color is obtained in about ten minutes. Consequently, in all the determinations of creatine and creatinine recorded below, ten minutes were allowed for the completion of the reaction. The solutions were then diluted and the readings taken immediately.

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<sup>1</sup> The experimental data are taken from the thesis presented by William C. Rose for the degree of Doctor of Philosophy, Yale University, 1911.

<sup>2</sup> Folin. *Amer Journ Physiol*, **xiii**, pp 45-65, 1905.

<sup>3</sup> Folin. *Zeitschr f physiol Chem*, **vi**, pp 223-42, 1904.

<sup>4</sup> Benedict and Myers. *Amer Journ Physiol*, **xviii**, pp 397-405, 1907.

*The influence of inanition on the creatine-creatinine excretion in rabbits, with some observations on the elimination of ammonia*

Four rabbits were allowed to starve until death resulted, the urines being analyzed for total nitrogen, ammonia nitrogen, total and preformed creatinine. The analytical data are summarized in Tables II to V. Throughout the experiments the animals were given water *ad libitum*. Creatine usually appeared in the urine on the second day, and progressively increased in actual amount until death. There was generally also an increase in the percentage of the total nitrogen present as creatine, but considerable variation is noted owing to the large increase in total nitrogen. *The elimination of nitrogen in the form of creatinine is remarkably constant*. Though slight fluctuations are noted—especially a tendency to decrease just before death—as in rabbits 1 and 3, the change is by no means commensurate with that observed in the creatine elimination. The percentage of creatinine-nitrogen invariably decreases as inanition progresses, on account of the increased total nitrogen elimination. These results agree with those of Dorner ('07).

**AMMONIA ELIMINATION** Incidentally it is of interest to note the elimination of ammonia nitrogen. So far as the writer is aware, no determinations of the ammonia excretion in rabbits during starvation have been previously published. It was expected that the percentage of the total nitrogen present in the form of ammonia would be increased, but such is not the case. With the exception of rabbit 1, where the results are very irregular, all animals excreted progressively smaller percentages of their nitrogen as ammonia. Usually, there is a tendency for the absolute amount to slightly increase before death, but this increase does not keep pace with the increase in the output of total nitrogen. Hence, the percentage steadily decreases.

No explanation can be given for the irregular results obtained with rabbit 1. As will be seen in the tabulated data (Table II), ammonia nitrogen was entirely absent, or present in amounts too small to be determined, on the 19th and 22d, but markedly increased until the 24th, when it represented 2.3 per cent of the total nitrogen. On the 25th and 26th it was still high, though the percentage had slightly fallen. This rabbit was a small animal,



TABLE IV  
Rabbit 3—Starvation

DATE	BODY WEIGHT	URINE						DIET, NOTES, ETC
		Volume	Reaction to litmus	Total V	Ammonia N	Creatinine N	Creatinine N	
	gms	cc		gms	mgms	mgms	per cent	
Dec 8	1815	134	Acid	1 15	74	27	13	No food
9								Urine for two days
10		35	Acid	0 81	33	15	14	No food
11		35	Acid	0 91	32	17	15	No food
12		50	Acid	1 31	20	18	15	No food
13		170	Acid	4 35	83	34	38	No food
14								Urine for two days
15		115	Acid	2 12	44	13	29	No food
16								Animal found dead in afternoon

TABLE V  
Rabbit 4—Starvation

DATE	BODY WEIGHT	URINE						DIET, NOTES, ETC
		Volume	Reaction to litmus	Total V	Ammonia N	Creatinine N	Creatinine N	
	gms	cc		gms	mgms	mgms	per cent	
Jan 15	1280	20*	Acid	0 18*	11*	0*	0*	No food
16		53	Acid	0 52	25	16	0	No food
17		60	Acid	0 99	20	18	16	No food
18								Two day's urine
19		74	Acid	1 24	20	15	31	No food
20		58	Acid	1 12	54	10	23	No food
21	800	92*	Acid	1 25*	10*	8*	30*	No food
								Animal died during night
								Not a full day's urine

\* Not a complete day's urine

scarcely full-grown, and was in very poor nutritive condition at the beginning of the experiment. Possibly this may explain the relatively large output of ammonia nitrogen in the last stages of inanition. It is interesting to note that the elimination of creatine nitrogen was greater in this animal than that observed in any other experiment, thus emphasizing the importance of the nutritive condition for the nitrogenous metabolism.

The fact that starvation does not induce an absolute or percentage increase in the elimination of ammonia nitrogen in rabbits, is quite in contrast with the findings of Brugsch,<sup>1</sup> Cathcart,<sup>2</sup> Grafe<sup>3</sup> and others on men. It would seem, therefore, that herbivora are either not subject to acidosis to the same extent that omnivora and carnivora are, or that they utilize bases other than ammonia for the neutralization of the acid products. In this connection, the suggestion of Burrige<sup>4</sup>—that creatine may serve as a neutralizing agent for lactic acid—is an interesting but unverified assumption.

In subsequent experiments upon rabbits the ammonia determinations were omitted.

*The influence of a carbohydrate diet upon the creatine-creatinine excretion in rabbits during inanition*

**DIET** Numerous attempts were made to give starving rabbits dextrose and sucrose by the stomach tube in solutions of varying strengths, but the results were invariably very unsatisfactory. Even when small doses of the sugar solutions were introduced into the stomach at intervals of several hours, diarrhoea was evoked, with resulting contamination of the urine. A more serious difficulty, however, was the fact that frequently the kidney excretion was practically stopped after giving the sugar for two or three days, and the animals died with symptoms of uremic poisoning. Hildebrandt<sup>5</sup> observed that large doses of dextrose

<sup>1</sup>Brugsch *Zeitschr f exp Path u Therap*, 1, pp 419-30, 1905

<sup>2</sup>Cathcart *Biochem Zeitschr*, vi, pp 122-23, 1907

<sup>3</sup>Grafe *Zeitschr f physiol Chem*, lxx, pp 21-52, 1910

<sup>4</sup>Burrige *Journ of Physiol*, xli, pp 303-04, 1910

<sup>5</sup>Hildebrandt *Zeitschr f physiol Chem*, xxxv, pp 141-52, 1902

exert a toxic action in rabbits fed upon a diet of oats. He attributed the toxicity to the production of large amounts of oxalic acid through the incomplete combustion of the sugar, and found that the addition of calcium carbonate to the diet neutralized the oxalic acid and prevented the appearance of abnormal symptoms. He makes no mention, however, of a decreased kidney excretion.

A typical experiment illustrative of this inhibition of kidney function, is summarized in Table VI. The sugar-feeding was begun on December 13. On the 15th, the animal had severe diarrhoea and contaminated most of the urine. On the 16th, the inability to excrete urine was very evident. The complete excretion for twenty-four hours was 10 cc, containing only 0.04 gram of total nitrogen. On the 17th, only 4 cc of urine were excreted. On the 18th, an attempt was made to increase the urine elimination by giving 100 cc of water by the stomach tube, but only 50 cc of a very dilute urine were obtained, containing 11 mgms of nitrogen as total creatinine. The 50 per cent retention of the water could not have been due to a depletion of the tissue moisture, for the animal had received water daily throughout the experiment. Retention is further indicated by the fact that the animal began to increase in weight on the 15th, and continued to increase until death on the 19th. Before death severe diarrhoea occurred, accompanied by a twitching of the neck and shoulder muscles, and followed by coma.

No explanation can at present be given of these observations. Out of some eight or ten experiments in which sugar was administered, satisfactory results were obtained only once (rabbit 6, Table VII), and this animal differed from the others in readily eating loaf-sugar, thus obviating the necessity of giving sugar solutions by the stomach tube.

In consequence of the difficulties associated with the sugar-feeding, this diet was abandoned. In the remaining experiments (Tables VIII to XI), soluble-starch suspended in water was given by the stomach tube with very satisfactory results. In no case did this diet interfere with kidney function.

**THE COMPOSITION OF THE URINE** In the following experiments, as in those previously described, creatine is a constant constituent of the urine of starving rabbits. In rabbit 6, creatine did

TABLE VI

*Rabbit 5—Starvation, carbohydrate feeding*

DATE	BODY WEIGHT	URINE						DIET, NOTES, ETC
		Vol ume	Specific gravity	Reaction to litmus	Total N	Creat- inine N	Crea- tine N	
Nov 30	gms 2240	cc 220	1 012	Alkaline	gms 0 67	mgms 30	mgms 0	Animal ate 300 gms carrots and 50 gms cracked corn
Dec 1	2260	210	1 014	Alkaline	0 47	27	0	Animal ate 300 gms carrots
2	2240	55	1 020	Alkaline	0 51	32	2	No food
3	2160	46	1 028	Acid	0 86	28	13	No food
4	2080	37	1 032	Acid	0 80	28	4	No food
5	2040	38	1 033	Acid	0 77	29	3	No food
6	1980	36	1 035	Acid	0 77	27	9	No food
7	1920	38	1 032	Acid	0 77	30	0	No food
8	1860	44	1 025	Acid	0 85	27	1	No food
9	1800	48	1 022	Acid	0 92	23	2	No food
10	1740	55	1 018	Acid	0 86	22	7	No food
11	1700	72	1 018	Acid	0 96	23	19	No food
12	1640	72	1 020	Acid	1 29	23	29	No food
13	1580	46	1 025	Acid	0 97	24	35	Animal was given 15 gms sucrose, (loaf sugar)
14	1510	21		Acid	0 26	22	16	30 gms sucrose by stomach - sound in 50 per cent sol in six equal doses
15	1550	2*		Acid	0 02*	3*	0*	35gms ditto in seven equal doses Part of a day's urine Ani- mal had diarrhoea
16	1600	10		Acid	0 04	3	1	25 gms ditto in five equal doses Com- plete kidney excre- tion for twenty-four hours
17	1620	4		Acid		2	trace	4 gms olive oil given by stomach-sound Complete kidney ex- cretion for twenty- four hours
18	1620	50	1 010	Neutral		7	4	No food 100 cc water given by stomach-sound
19	1660							Animal died in after- noon with severe diarrhoea

\*Not a complete day's urine see notes

not appear in appreciable amounts until after a surprisingly long period of inanition. Small amounts were periodically excreted from February 7 to March 1, but the quantities were too small to be of any significance. For ten days, beginning with February 14th, the animal was given loaf-sugar each day, but the only effect observed was a decrease in the total nitrogen elimination, with a corresponding increase in the percentage of creatinine nitrogen. On February 25, starvation was resumed, and creatine first appeared in significant amount on March 2.

This remarkably long period of starvation was undoubtedly made possible by the excellent nutritive condition of the animal, it having been fed liberally for several weeks before beginning the experiment. The store of glycogen probably prevented the appearance of much urinary creatine, until the liver and muscles had had their glycogen supply nearly depleted.

On March 2, sugar-feeding was again begun with the result that the creatine nitrogen fell from 14 mgms on the 2d, to zero on the 5th. After two days of fasting the creatine nitrogen again increased until it was more than twice as large in amount as the creatinine nitrogen. On resuming the sugar diet, however, it was rapidly reduced to zero. On March 13, an attempt was made to give a protein diet consisting of coagulated egg-white, but diarrhoea resulted, followed by the death of the animal on the next day.

Results similar to these were obtained on rabbits 7, 8 and 9, each of which received the soluble-starch diet instead of the sugar. The creatine elimination was reduced at will by giving an absolutely nitrogen- and fat-free carbohydrate diet. Usually the amount of total nitrogen and creatine nitrogen increased the first day of the carbohydrate administration, but rapidly decreased on continuing the feeding. *Total nitrogen and total creatinine appear to have a common source*, for an increase or decrease in the latter is always accompanied by a similar change in the former. The significance of this will be discussed later.

The experiment upon rabbit 10 is particularly interesting because of the results following the *administration of alcohol*. The protein-sparing effect of small doses of alcohol is well known.<sup>1</sup>

<sup>1</sup>For the literature on this subject cf. Rosemann *Oppenheimer's Handbuch der Biochemie*, iv, part 1, p. 433, 1903.



TABLE VII  
Rabbit 6—Starvation, carbohydrate feeding

DATE	BODY WEIGHT	URINE						DIET, NOTES, ETC
		Volume	Reaction to litmus	Total N	Creatinine N	Creatinine	Creatinine	
	gms	cc		gms	mgms	mgms	per cent	
Feb 5	2240	72	Alkaline	0.52	34	0	6.5	No food
6	2140	30	Acid	0.79	34	0	4.3	No food
7	2140	54	Acid	0.89	33	6	3.7	No food
8	2060	35	Acid	0.81	36	1	4.4	No food
9	2000	24	Acid	0.72	32	2	4.4	No food
10	1960	30	Acid	0.83	36	2	4.3	No food
11	1940	26	Acid	0.78	32	0	4.1	No food
12	1880	36	Acid	0.82	35	0	4.3	No food
13	1880	29	Acid	0.77	36	0	4.7	No food
14	1820	29	Acid	0.59	33	4	5.6	Animal ate 22 grams loaf sugar (sucrose)
15	1820	82	Acid	0.33	33	0	10.0	Animal ate 23 grams loaf sugar (sucrose)
16	1840	30	Acid	0.24	26	3	10.8	Animal ate 19 grams loaf sugar (sucrose)
17	1800	16	Acid	0.27	32	0	11.8	Animal ate 20 grams loaf sugar (sucrose)
18	1780	16	Acid	0.24	31	0	12.8	Animal ate 17 grams loaf sugar (sucrose)
19	1760	18	Acid	0.26	32	0	12.3	Animal ate 21 grams loaf sugar (sucrose)
20	1760	18	Acid	0.23	33	0	11.7	Animal ate 20 grams loaf sugar (sucrose)
21	1740	16	Acid	0.22	28	0	12.7	Animal ate 17 grams loaf sugar (sucrose)
22	1740	16	Acid	0.22	30	0	13.6	Animal ate 23 grams loaf sugar (sucrose)
23	1720	15	Acid	0.27	32	1	11.9	Animal ate 23 grams loaf sugar (sucrose)
24	1720	14	Acid	0.24	26	2	10.8	Animal ate 19 grams loaf sugar (sucrose)

25	1700	7	Acid	0.04	13	0	32.5	0	No food	Twelve hours' urine, 9 a m to 9 p m
		8	Acid	0.19	13	0	7.0	0	No food	Twelve hours' urine, 9 p m to 9 a m
		12	Acid	0.30	11	1	4.7	0.3	No food	Twelve hours' urine, 9 a m to 9 p m
26	1720	15	Acid	0.31	12	1	3.5	0.3	No food	Twelve hours' urine, 9 p m to 9 a m
27	1080	38	Acid	0.87	30	0	3.1	0	No food	
28	1010	38	Acid	0.86	21	0	2.8	0	No food	
Mar										
1	1600	51	Acid	1.01	23	0	2.2	0.6	No food	Twelve hours' urine, 9 a m to 9 p m
		10	Acid	0.78	11	8	1.8	1.0	No food	
2	1560	30	Acid	0.75	13	6	1.7	0.8	Animal ate 20 grams loaf sugar (sucrose)	Twelve hours' urine, 9 p m to 9 a m
									Animal ate 29 grams loaf sugar (sucrose)	
3	1560	28	Acid	0.63	23	4	3.7	0.6	Animal ate 21 grams loaf sugar (sucrose)	
4	1510	14	Acid	0.29	22	1	7.6	0.3	Animal ate 2 grams loaf sugar (sucrose)	
5	1520	12	Acid	0.28	19	0	6.8	0	No food	
6	1560	31	Acid	0.84	19	1	2.3	0.5	No food	
7	1490	102	Acid	1.80	23	37	1.2	2.0	No food	
8	1420	90	Acid	1.82	21	53	1.3	2.0	Animal ate 24 grams loaf sugar (sucrose)	
9	1380	20	Acid	0.37	17	9	1.6	2.1	Animal given 20 grams sucrose (50 per cent solution) by stomach-sound	
10	1100								Animal given 23 grams sucrose (50 per cent solution) by stomach-sound	
									Diarrhoea	Urine (contaminated No analyses made)
									Animal ate 1 grams loaf sugar	Very weak
11	1380	10	Acid	0.41	18	1	1.4	0.2	No food	
12	1310	104	Acid	1.10	19	26	1.1	1.8	Animal ate 28 grams coagulated egg-white	
13	1225	100	Acid	1.66	13	75	0.8	1.5	Diarrhoea	Animal refused to eat egg-white and sucrose
11	1180								Very weak	Died in afternoon with severe diarrhoea

TABLE VIII  
Rabbit 7—Starvation, carbohydrate feeding

DATE	BODY WEIGHT	URINE						DIET, NOTES, ETC
		Volume	Specific gravity	Reaction to litmus	Total N	Creatinine	Creatinine	
	gms	cc			gms	mgms	per cent	
Oct 6	2080	204	1 014	Alkaline	0 51	34	0	Mixed diet of oats, cracked corn and carrots
7	2000	190	1 020	Alkaline	0 83	37	5	Diet of carrots
8	1920	182	1 017	Alkaline	0 86	37	11	Mixed diet of oats, cracked corn and carrots
9	1900	110	1 029	Acid	0 68	30	5	Mixed diet of oats, cracked corn and carrots
10	1920	45	1 027	Alkaline	0 81	34	7	No food
11	1840	44	1 028	Acid	0 89	30	13	No food
12	1800	50	1 028	Acid	1 22	33	30	No food
13	1760	52	1 028	Acid	1 28	29	40	No food
14	1700	93	1 015	Acid	1 32	30	44	No food
15	1670	78	1 016	Acid	0 83	31	23	20 grams soluble starch suspended in 80 cc water
16	1700	30	1 011	Acid	0 30	26	37	30 grams soluble starch suspended in 110 cc water
17	1720	32	1 014	Acid	0 51	26	0	30 grams soluble starch suspended in 70 cc water
18	1660	105	1 019	Acid	1 89	29	5	No food
19	1510	94	1 020	Acid	1 83	28	46	No food
20	1480	*22	1 015	Acid	0 33*	5*	70	30 grams soluble starch suspended in 75 cc water
							20*	37 grams soluble starch suspended in 70 cc water
							1 5	Animal had slight diarrhoea contaminating part of urine
21	1460	50	1 020	Acid	0 80	21	2 6	25 grams soluble starch suspended in 70 cc water
22	1420						4 3	Experiment stopped Animal has severe diarrhoea

\* Not complete day's urine see notes

TABLE IX  
Rabbit 8—Starvation, carbohydrate feeding

DATE	BODY WEIGHT gms	URINE						Diet notes etc
		Volume cc	Specific gravity	Reaction to litmus	Total N gms	Creatinine mgms	Creatinine mgms	
Oct 6	1820	78	1.017	Alkaline	0.57	25	0	Mixed diet of carrots and oats
7	1780	116	1.020	Alkaline	0.75	28	1.1	Mixed diet of carrots and oats
8	1710	130	1.019	Alkaline	0.81	28	3.7	Mixed diet of carrots and oats
9	1700	71	1.027	Alkaline	0.68	20	3.5	Mixed diet of carrots and oats
10	1720	58	1.020	Acid	0.90	32	3.8	No food
11	1620	50	1.027	Acid	1.03	28	0	No food
12	1600	50	1.026	Acid	1.22	29	2.7	No food
13	1560	52	1.030	Acid	1.32	28	2.1	No food
14	1500	52	1.033	Acid	1.60	27	2.1	No food
15	1460	115	1.020	Acid	1.85	28	1.7	20 grams soluble starch suspended in 70 cc water
16	1400	45	1.018	Acid	0.75	23	1.5	30 grams soluble starch suspended in 70 cc water
17	1380	60	1.010	Acid	0.35	21	3.1	30 grams soluble starch suspended in 70 cc water
18	1386	30	1.018	Acid	0.55	5	0.9	No food
19	1310	82	1.024	Acid	1.81	25	1.5	No food
20	1210	*56	1.020	Acid	1.08*	18*	1.1	10 grams soluble starch suspended in 85 cc water
						12*	1.7	Animal died during day Diarrhoea before death

\* Not complete day's urine, see notes

TABLE X  
Rabbit 9—Starvation, carbohydrate feeding

DATE	BODY WEIGHT	URINE						DIET, NOTES, ETC
		Volume	Specific Gravity	Reaction to litmus	Total N	Creatinine N	Creatinine N	
	gms	cc			gms	mgms	Per cent per cent	
Oct 23	2420	160	1 017	Alkaline	0 63	35	0	Mixed diet of carrots and oats
24	2340	134	1 020	Alkaline	0 63	40	0	Mixed diet of carrots and oats
25	2360	292	1 015	Alkaline	0 86	41	0	Mixed diet of carrots and oats
26	2380	88	1 015	Alkaline	0 69	35	2	No food
27	2280	100	1 014	Acid	1 10	33	6	No food
28	2220	130	1 010	Acid	0 90	26	9	No food
29	2220	175	1 011	Acid	1 46	43	2	No food
30	2120	120	1 012	Alkaline	1 28	31	19	10 grams soluble starch suspended in 35 cc water
31	2080	80	1 020	Alkaline	1 23	30	17	20 grams soluble starch suspended in 70 cc water
Nov 1	2030	70	1 017	Alkaline	0 90	31	3	30 grams soluble starch suspended in 90 cc water
2	2060	142	1 012	Alkaline	1 34	31	14	No food
3	2000	160	1 017	Alkaline	2 66	32	48	No food
4	1840	140	1 014	Acid	2 01	30	60	40 grams soluble starch suspended in 100 cc water
5	1820	58	1 015	Acid	0 53	30	9	45 grams soluble starch suspended in 100 cc water
6	1820	66	1 009	Alkaline	0 17	28	0	45 grams soluble starch suspended in 90 cc water
7	1880	34	1 016	Acid	0 16	22	2	No food
8	1820	56	1 014	Alkaline	0 51	19	3	No food
9	1760	50	1 016	Alkaline	0 99	19	31	No food
10	1610							Animal was very weak Experiment was stopped

Recently, Kochmann and Hall<sup>1</sup> have reported the results of experiments in which they found that small doses, given subcutaneously, greatly prolonged the lives of starving rabbits. In consideration of these results it seemed possible that alcohol might exert an influence on the creatine excretion similar to that produced by carbohydrates. Hence, in the experiment upon rabbit 10, alcohol was given by the stomach-sound for two days. Rather large doses were necessary in order to make the experiment comparable in fuel value with those in which carbohydrates were fed. On the 17th (Table XI), 12 cc of absolute alcohol, well diluted with water, were given in divided dose several hours apart. The animal was rendered intoxicated for two or three hours after each dose. The urine analysis for that day shows that the total nitrogen was increased, while the creatine nitrogen was more than doubled. On the 18th, the animal was kept intoxicated practically all day by the frequent administration of alcohol, with the result that while the creatine nitrogen was again increased, the creatinine and total nitrogen were slightly decreased. Here again the changes in *total creatinine* elimination are associated with changes in the same direction in total nitrogen elimination.

That the increase in creatine nitrogen was not due to the alcohol *per se* seems probable from the observations of Mendel and Hilditch ('10), who found no increase in creatine elimination in dogs even after prolonged administration of alcohol. The increase must therefore have been due solely to the continued lack of carbohydrates. At the same time, it must be admitted that the doses of alcohol given to the rabbit were presumably sufficient to produce the so-called toxic nitrogenous catabolism.

On the 19th, administration of starch suspensions was begun. Only 15 grams of soluble-starch were given the first day, which were not sufficient to prevent the creatine and total nitrogen from again increasing. On the 20th and 21st, 35 and 40 grams respectively, of starch were given, with the result that the creatine elimination rapidly disappeared, accompanied by an enormous decrease in total nitrogen excretion. Starvation and starch-feeding were again repeated on this animal with results similar to those previously described.

<sup>1</sup>Kochmann and Hall *Pflüger's Archiv*, cxxvii, pp 280-356, 1909

TABLE XI  
Rabbit 10—Starvation alcohol, carbohydrate feeding

DATE	BODY WEIGHT	URINE							DIET, NOTES ETC
		Volume	Specific Gravity	Reaction to litmus	Total N	Creatinine N	Creatinine N	Creatinine N	
	gms	cc			gms	mgms	per cent	per cent	
Nov									
11	2480	230	1 020	Alkaline	1 09	41	0	3 8	Mixed diet of carrots and oats
12	2400	106	1 015	Acid	0 76	38	4	5 0	No food
13	2250	50	1 030	Acid	0 82	32	7	3 9	No food
14	2180	38	1 032	Acid	1 06	36	14	3 4	No food
15	2120	46	1 032	Acid	1 18	35	13	3 0	No food
16	2060	54	1 027	Acid	1 16	34	18	2 9	No food
17	2000	130	1 012	Acid	1 31	33	41	2 5	No food
18	1920	167	1 008	Acid	1 14	24	47	2 1	12 cc abs alcohol in 60 cc water doses Given in two equal
19	1860	130	1 021	Acid	1 44	24	73	1 7	21 cc abs alcohol in 100 cc water doses Given in four equal
20	1740	90	1 017	Acid	1 24	25	33	2 0	8 p m 15 grams soluble starch suspended in 25 cc water
21	1700	42	1 013	Acid	0 30	16	0	5 3	35 grams soluble starch suspended in 70 cc water
22	1800	30	1 013	Acid	0 26	19	6	7 3	40 grams soluble starch suspended in 95 cc water
23	1740	102	1 011	Acid	1 27	34	29	2 7	No food
24	1610	70	1 022	Acid	1 50	31	68	2 1	No food
25	1600	70	1 020	Acid	1 21	24	41	2 0	15 grams soluble starch suspended in 25 cc water
26	1620	77	1 014	Acid	0 96	26	23	2 7	40 grams soluble starch suspended in 95 cc water
27	1640	52	1 020	Acid	1 01	21	33	2 1	35 grams soluble starch suspended in 75 cc water No food

From the foregoing experiments, it can be definitely stated that *a carbohydrate diet absolutely nitrogen- and fat-free, produces a marked reduction in the elimination of total nitrogen and creatine in starving rabbits*. If the carbohydrate feeding is continued for two or three days creatine disappears. *The administration of alcohol produces no decrease in urinary creatine or nitrogen*.

*The influence of fat and protein upon the creatine-creatinine excretion in rabbits during inanition*

Five experiments were made to determine the effect of fat and protein feeding upon the creatine-creatinine elimination in starving rabbits. The fat diet consisted of an emulsion of 73 per cent peanut oil, 2 per cent lecithin, and 25 per cent water<sup>1</sup>. Small doses of the emulsion were given at intervals of several hours by the stomach-sound. The proteins used were casein and egg-white. The results of the experiments will be seen in Tables XII to XVI.

In rabbits 11, 12 and 13, the influence of the *fat diet without protein* was investigated. With the exception of animal 12, absolutely no reduction in creatine or total nitrogen followed the fat administration. In this animal, agar-agar was also fed with the fat emulsion to prevent diarrhoea. Hoffmann<sup>2</sup> has recently shown that agar-agar can be decomposed and absorbed by rabbits, but does not increase the sugar in the urine when the animals are phlorhizinized. He believes that it is converted into fatty acids and absorbed in this form. There is no reason for assuming that fatty-acids arising from the fermentation of agar would behave differently, in regard to their influence on the creatine elimination, from those liberated in fat digestion. Still it is interesting to observe that only in the animal that received agar did a decrease in creatine and nitrogen elimination follow the fat feeding.

It is impossible to state the degree of utilization of the fat diet. Diarrhoea frequently occurred and probably resulted in poor absorption on these days. Herbivora are, furthermore, susceptible to lipuria after the ingestion of large amounts of fat. In order to ascertain definitely the amount of fat actually metabo-

<sup>1</sup>This was prepared for the laboratory by Fairchild Brothers and Foster, New York.

<sup>2</sup>Hoffmann *Inaug Diss*, Halle, pp 27, 1910



TABLE XII  
Rabbit 11—Starvation, fat feeding

DATE	BODY WEIGHT	URINE							DIET, NOTES, ETC
		Volume	Specific Gravity	Reaction to litmus	Total N	Creatinine mgms	Creatine mgms	Creatinine per cent	
Mar 8	1900	74	1 025	Acid	1 24	27	6	0 5	No food
9	1800	76	1 024	Acid	1 49	26	13	2 2	No food
10	1750	88	1 034	Acid	2 23	31	35	1 7	No food
11	1680	120	1 026	Acid	2 78	32	68	1 4	16 cc fat emulsion in 2 doses
12	1530	80*	1 027	Acid	1 93*	23*	50*	1 2	23 cc fat emulsion in 3 doses
13	1400	58*	1 026	Acid	1 37*	16*	31*	2 6	urine was contaminated
14	1280	50*	1 026	Acid	1 19*	15*	50*	2 3	No food Severe diarrhoea continued
15	1160							4 2	day's urine
								1 3	25 cc fat emulsion in 4 doses
									Not complete day's urine
									Animal died

\* Not complete day's urine, see notes

TABLE VIII  
Rabbit 12—Starvation, fat, carbohydrate feeding

DATE	BODY WEIGHT	URINE							DIET NOTES, ETC
		Volume	Specific Gravity	Reaction to litmus	Total N	Creatinine N mgms	Creatinine N mgms	Creatinine N percent	
Feb	gms	cc			gms				
16	2060	54	1 019	Acid	0 53	33	0	0 2	No food
17	2000	100	1 018	Acid	1 58	61	0	3 9	No food
18	1980								Urine for two days
19	1920	48	1 023	Acid	0 86	26	7	3 0	No food
20	1860	52	1 020	Acid	0 70	26	1	3 3	No food
21	1780	72	1 015	Acid	0 83	27	4	3 3	No food
22	1760	48	1 023	Acid	0 71	26	0	3 7	No food
23	1700	53	1 024	Acid	1 07	31	11	2 9	No food
24	1640	55	1 025	Acid	1 25	29	11	2 3	No food
25	1580	70	1 021	Acid	1 51	27	26	1 8	10 cc fat emulsion in two doses
26	1520	84	1 016	Acid	0 97	27	10	2 8	26 cc fat emulsion in four doses
27	1480	50	1 020	Acid	0 79	25	0	3 2	Agar-agar 10 grams sol starch in 25 cc water
28	1420	50	1 026	Acid	0 94	23	14	2 4	No food
Mar									
1	1390	105	1 020	Acid	1 75	23	43	1 3	No food
2	1320	70*	1 019	Acid	1 38*	17*	83*	1 2	28 cc fat emulsion in three doses
3	1280	38*	1 012	Acid	0 29*	6*	12*	2 1	19 cc fat emulsion in three doses Slight diarrhoea Animal died in evening Urine for about eleven hours

\* Not complete day's urine, see notes

TABLE XIV  
Rabbit 13—Starvation, fat feeding

DATE	BODY WEIGHT	URINE						Creatinine N	Creatinine N	Diet, notes, etc
		Volume	Specific Gravity	Reaction to litmus	Total N	Creatinine N	Creatinine N			
	gms	cc			gms	mgms	mgms	per cent	per cent	
Mar 23	2380	34	1 032	Acid	0 70	28	0	4 0	0	No food
24	2340	47	1 025	Acid	1 07	34	0	3 2	0	No food
25	2300	240	1 007	Acid	1 89	63	0	3 3	0	No food
26	2220									
27	2160	45	1 022	Acid	0 72	26	4	3 6	0 6	No food
28	2120	80	1 016	Acid	0 98	34	0	3 5	0	No food
29	2100	35	1 027	Acid	0 83	30	7	3 6	0 8	No food
30	2020	34	1 032	Acid	0 96	30	10	3 1	1 0	No food
31	1980	70*	1 008	Acid	0 52*	13*	7*	2 5	1 3	No food Not complete day's urine Bladder was not squeezed out
Apr 1	1980	92†	1 032	Acid	2 38†	46†	49†	1 9	2 1	No food More than a day's urine Part of urine of 31st
2	1900	42*	1 010	Acid	0 69*	9*	11*	1 3	1 6	23 cc fat emulsion in three doses Diarrhoea Not complete day's urine
3	1820	74*	1 017	Acid	0 62*	16*	34*	2 6	5 5	23 cc fat emulsion in three doses Diarrhoea Not complete day's urine Experiment stopped

\* Not complete day's urine, see notes

† More than a day's urine see notes

lized, it would therefore be necessary to determine the respiratory quotient. It is certain, however, that *the creatine output cannot be reduced in rabbits by feeding an exclusive fat diet*. These results are in accord with the conclusions of Cathcart ('09) in fasting men.

The influence of a *mixed protein and fat diet* was tested in rabbits 14 and 15. In the former, from the 12th to the 14th, an exclusively protein diet was fed, but it was found impractical to give sufficient calories. The whites of eight eggs are necessary in order to yield an amount of energy equivalent to 40 grams of carbohydrate. It is out of the question to feed so large an amount of protein to a rabbit without producing diarrhoea. Hence, from the 15th to the 18th, sufficient nitrogen was given in the form of protein to cover the nitrogenous waste of the animal, and the caloric value was raised by the addition of fat emulsion. *No reduction in the creatine elimination occurred on this diet in the absence of carbohydrates*. On the 19th, a carbohydrate diet was substituted for the protein-fat diet, with the result that the outputs of creatine and nitrogen subsequently decreased, notwithstanding the fact that the animal was very weak and emaciated. Similar results with the fat-protein feeding were obtained in rabbit 15. On account of diarrhoea it was impossible to continue the carbohydrate diet at the end of this experiment sufficiently long to reduce the creatine.

The results obtained after protein feeding are in striking contrast to those briefly reported by Osterberg and Wolf ('08). These investigators state that in the dog, "the creatine produced by starvation is inhibited by very small amounts of ingested protein." Since no figures are given in the preliminary report, it is not apparent what "very small amounts" denote. It is true that in rabbits 14 and 15 the protein ingested was calorifically insufficient, but the amount of nitrogen which it yielded was more than necessary to compensate the nitrogenous waste of the animal. That the protein was absorbed is shown by the greatly increased urinary nitrogen. In view of Osterberg and Wolf's results, a decrease in creatine might be expected, but such was not obtained. In experiments upon fasting men, Cathcart ('09) found that the addition of protein produced no alteration in the creatine output on a fat diet. Since this is true for herbivora and omnivora, it seems unlikely—in the absence of more definite data—that carnivora differ so radically in their metabolism.

TABLE XV  
Rabbit 14—Starvation, protein-fat, carbohydrate feeding

DATE	BODY WEIGHT	URINE							DIET NOTES ETC
		Volume	Specific gravity	Reaction to litmus	Total N	Creatinine mgms	Creatine mgms	Creatinine percent	
Mar 10	1980	84	1 025	Acid	1 36	35	3	2 6	No food
11	1860	70	1 025	Acid	1 24	31	24	2 5	No food
12	1760	110	1 022	Acid	2 08	31	44	1 5	10 grams dry casein in two doses
13	1700	220	1 015	Acid	3 33	28	47	0 8	6 grams casein in one dose White of one egg in two doses
14	1600	140	1 027	Acid	3 06	26	43	0 8	Whites of three eggs in four doses
15	1520	58*	1 033	Acid	1 51*	11*	24*	0 7	Whites of one and one-half eggs and 23 cc fat emulsion given in three doses Diarrhoea About twelve hours, urine
16	1440	90	1 030	Acid	1 87	26	46	1 4	No food until evening when 5 grams of nutrose were given
17	1360	74	1 021	Acid	1 43	24	46	1 7	White of one egg and 20 cc fat emulsion given in four doses
18	1340	94	1 020	Acid	1 38	21	47	1 5	Same diet as on 17th
19	1340	70	1 020	Acid	1 01	16	44	1 6	30 grams soluble starch given in three doses
20	1320	58	1 015	Acid	0 62	19	16	3 1	40 grams soluble starch given in four doses
		{ 62*	1 011	Acid	0 38*	9*	5*	2 4	30 grams soluble starch given in three doses Urine for about thirteen hours
21	1340	{ 43*	1 011	Acid	0 28*	6*	10*	2 1	No food Animal very weak Experiment stopped Urine for eleven hours

\*Not complete day's urine see notes

TABLE XVI  
*Rabbit 15—Starvation, protein-fat feeding*

DATE	BODY WEIGHT	URINE								DIET NOTES, ETC
		Volume	Specific gravity	Reaction to litmus	Total N	Creatinine N	Creatinine N	Creatinine N		
									cc	
Mar 8	1860	47	1 025	Acid	0 83	20	3	3 1	0 1	No food
9	1780	62	1 016	Acid	0 85	23	4	2 7	0 5	No food
10	1760	44	1 018	Acid	0 85	31	3	3 6	0 4	No food
11	1720	54	1 019	Acid	0 89	31	14	3 5	1 6	No food
12	1720	45	1 025	Acid	0 80	28	15	3 2	1 7	No food
13	1650	55	1 016	Acid	0 90	25	15	2 8	1 7	No food
14	1600	42	1 025	Acid	0 98	26	15	2 7	1 5	No food
15	1540	42	1 032	Acid	1 14	20	16	1 8	1 4	No food
16	1500	110	1 015	Acid	1 43	21	20	1 7	1 1	White of one egg and 20 cc fat emulsion given in four doses
17	1500	90	1 020	Acid	1 42	21	27	1 7	1 0	Same diet as on 16th Animal had diarrhoea, but urine was not contaminated
18	1420	86*	1 020	Acid	1 02*	18*	19*	1 8	1 0	Same diet as on 16th Diarrhoea continues Not complete day's urine
19	1340	86*	1 019	Acid	1 16*	11*	29*	1 2	2 5	20 grams soluble starch Severe diarrhoea Not complete day's urine Animal very weak
20										Experiment stopped Animal very weak Severe diarrhoea

\*Not complete day's urine, see notes

The want of available carbohydrate seems to be the primary factor that induces the appearance of urinary creatine in starvation. It is interesting in this connection to note that creatine is also excreted in many conditions where carbohydrates are not properly oxidized. Reference has already been made to the constant presence of creatine in the urine during diabetes mellitus. Recently Krause and Cramer ('10) have demonstrated the elimination of creatine in dogs, following the injection of phlorhizin. Similar experiments were made by Cathcart and Taylor ('10). The latter investigators fed their animals a creatine-free diet and found that no creatine was excreted before the glycosuria, provided there was an adequate supply of carbohydrates. If the supply was very small, creatine appeared just as in starvation. These results are, of course, in accord with the findings of the present investigations on fasting rabbits.

After a small carbohydrate intake, Cathcart and Taylor ('10) observed an output of creatine as the result of the injection of phlorhizin. The creatine was present only so long as the glycosuria persisted. When, however, the supply of ingested carbohydrates was abundant, in spite of the glycosuria, creatine was not excreted. After substituting fat for the carbohydrate of the diet, the injection of phlorhizin was followed by a much less intense glycosuria, but by a marked excretion of creatine.

It would appear then that fat in the food, even in considerable amount, does not prevent protein catabolism, or in other words cannot replace carbohydrates under the experimental conditions of Cathcart and of the writers. It is possible, and indeed probable that fat may replace a certain amount of carbohydrate, provided there is still sufficient carbohydrate in the diet to exert a regulatory influence over certain essential metabolic processes. This conception is in accord with Rosenfeld's<sup>1</sup> view of a specific action of carbohydrates on fat metabolism. This author believes that fats cannot be properly burned unless carbohydrates are present. Similar views were formulated by Landergren,<sup>2</sup> who found that with a nitrogen intake of approximately one gram, the output

<sup>1</sup>Rosenfeld *Verhandl. d. xxiv Kongres. f. inner. Med.*, Wiesbaden, pp. 279-83, 1907.

<sup>2</sup>Landergren *Skand. Arch. f. Physiol.*, iv, pp. 112-75, 1903.

of urinary nitrogen could be reduced to a minimum (3 to 4 grams per day) by feeding large amounts of carbohydrates. If, on the other hand, the carbohydrates of the diet were replaced by fats the nitrogen excretion decreased the first day, but increased on the second and third days. Landergren believes this increase to be due, not to a specific dynamic action of the fat, but to a depletion of the store of glycogen. In the absence of carbohydrates the fats are unable to exert their protein-sparing effect. It may be equally true that creatine cannot be properly catabolized, or converted into creatinine in the absence of carbohydrate food, or that the cell processes themselves are radically different under such conditions.

So we might expect disturbances of the liver to lead to the production of creatine through improper glycogenic function, and in fact such is the case. Mellanby ('07) and van Hoogenhuyze and Verploegh ('08) observed the excretion of creatine in hepatic diseases, notably carcinoma, and Underhill and Kleiner ('08) found an increased excretion of creatine in the urines of dogs poisoned with hydrazine—a substance which is known to have a specific action upon the cytoplasm of the parenchymatous cells of the liver. The internal administration of chloroform which, as Clark<sup>1</sup> has shown, produces great degenerative changes in the hepatic cells, induces the appearance of creatine in the urine (cf Howland and Richards, '09, and Lindsay, '11). More recently van Hoogenhuyze and ten Doeschate ('11) have reported the presence of large amounts of creatine in eclampsia. Likewise, London and Boljarski ('09), Foster and Fisher ('11), and others, found creatine in the urine of Eck fistula dogs, where the hepatic functions are entirely removed.

It is true that the presence of creatine in these conditions may be explained by assuming that the liver is the organ that brings about the conversion of creatine into creatinine, and that when the hepatic cells are diseased or removed from the sphere of action by artificial alterations of the circulation, this conversion cannot occur. But if this assumption were correct, creatine alone should be excreted in animals with Eck fistulas, and creatinine should be entirely absent—a condition which has never been attained.

<sup>1</sup>Clark *Proc Roy Soc Edinb*, xxix, pp 418-26, 1909



Van Hoogenhuyze and ten Doeschate ('11) attempt to explain the presence of creatine in eclampsia on the ground that the liver is diseased, and is, therefore, unable to bring about the dehydration. As evidence of the liver disorder they refer to the *post mortem* findings of capillary hemorrhages in the liver parenchyma, and to the greatly reduced tolerance for sugar in such patients. May not the reduced tolerance, and hence the lowered glycolytic function, be the factor occasioning the excretion of creatine, rather than the condition of the liver *per se*? The presence of creatine in the urine during pregnancy as noted by Murlin ('08-'09), Longridge,<sup>1</sup> and others, can readily be explained on this assumption, for Bar<sup>2</sup> found a greatly reduced tolerance for sugar in pregnant women. Certainly, from a consideration of the data which have been collected in regard to the effect of carbohydrates on creatine elimination, it is just as probable that the real factor in the production of creatine in hepatic disease is a disturbance of carbohydrate metabolism.

To further test the influence of the amount of glycogen and of the condition of the hepatic cells upon creatine excretion, two experiments were undertaken upon dogs. In the first, an attempt was made to remove the glycogen by repeated injections of *phlorhizin* in a starving animal. It was hoped that after repeated *phlorhizin* administration the glycogen could be largely removed from the animal, with the result that creatine would continue to be present in the urine after the glycosuria had disappeared. In the second experiment a fasting animal was given frequent injections of *phosphorus oil*, in order to cause degeneration of the hepatic cells. The protocols are summarized in Tables XVII and XVIII.

The *phlorhizinized* animal received the drug subcutaneously in sodium carbonate solution, according to the procedure recommended by Lusk.<sup>3</sup> Three doses of 2 grams each were given on January 20 and 21, and one dose of 2 grams on the 22d. Sugar continued to be excreted until January 27, whereupon the creatine estimations were begun after the glycosuria ceased. As will be seen, creatine alternately appeared and disappeared. On

<sup>1</sup>Longridge. Cited by van Hoogenhuyze and ten Doeschate ('11)

<sup>2</sup>Bar (cited by Lequeux) *L'Obstetrique*, III, p. 506, 1910

<sup>3</sup>Lusk. *Amer Journ of Physiol*, XII, pp. 164-65, 1908

TABLE XVII  
Dog 16—Phlorhizin and starvation

DATE	BODY WEIGHT kilos	URINE							REMARKS
		Volume cc	Specific Gravity	Reaction to litmus	Total N gms	Creatinine N	Creatinine N	Creatinine N	
						mgms	mgms	percent	
Jan									
27	10.3	110	1.030	Acid	2.85	83	0	2.9	0
28	10.2	190	1.020	Acid	2.91	77	0	2.6	0
29	10.0	Urine	contaminated						
30	9.8	Urine	contaminated						
31	9.5	180	1.019	Acid	3.09	36	20	1.2	0.6
Feb									
1	9.2	90	1.025	Acid	2.31	31	34	1.3	1.5
2	9.0	100	1.030	Acid	2.40	48	7	2.0	0.3
3	9.0	120	1.030	Acid	3.42	63	0	1.8	0
4	8.9	60	1.045	Acid	2.26	65	0	2.9	0
5	8.9	100	1.035	Acid	2.31	20	6	1.3	0.3
6	8.8	160	1.040	Acid	4.20	34	0	0.8	0
7	8.6	100	1.025	Acid	3.15	31	0	1.0	0
8	8.1	90	1.035	Acid	3.45	50	0	1.4	0
9	8.1	100	1.030	Acid	3.96	58	0	1.5	0
10	7.9	130	1.035	Acid	5.32	43	6	0.8	0.1
11	7.9	120	1.040	Acid	4.96	41	25	0.8	0.5
12	7.7	150	1.035	Acid	6.30	28	63	0.1	1.0
13	7.3	Urine	contaminated						
14	7.3	50	1.030	Acid	4.62				

Animal very weak. Experiment stopped

\*The ammonium salts were given in connection with other investigations and have no relation to the present work

February 1, the amount of creatine nitrogen exceeded the creatinine nitrogen. Two days later creatine was entirely absent. On February 10, it was again excreted and continued to be present in increasing amount until February 12, when it exceeded the amount of preformed creatinine by over 200 per cent. Unfortunately, the urines of the next two days were contaminated with feces, and analyses were not made.

No adequate explanation of this irregular excretion of creatine is apparent. It is probable that the amount present was due to the fasting, though the injection of phlorhizin may have been a contributing factor in the partial removal of glycogen. In regard to the disappearance and subsequent reappearance of creatine, the observation of Pflüger and Junkersdorf<sup>1</sup>—that a re-formation of glycogen from protein occurs in phlorhizinized dogs—is of particular interest. If we accept this as an established fact, the sequence of changes in creatine elimination can be readily understood. The phlorhizin and starvation removed sufficient glycogen to leave the animal in need of carbohydrate, and creatine immediately appeared in significant amount. A re-formation of glycogen caused the subsequent disappearance of creatine, which did not again appear until the glycogen supply had again been depleted on February 10. Of course this explanation is entirely theoretical and tentative. It is well known that dogs are especially hard to render glycogen-free. They seem to retain or replenish at least part of their store, in spite of varied experimental attempts to exhaust it. This fact may explain their ability to endure fasting usually for remarkably long periods without serious consequences,<sup>2</sup> and without the excretion of large amounts of creatine or nitrogen.

In the experiment on the phosphorus-poisoned dog (Table XVIII), creatine was constantly present in the urine in significant amounts. Beginning with May 28 and continuing until the death of the animal, the amount of nitrogen excreted in this form greatly exceeded that as creatinine. This appearance of creatine in con-

<sup>1</sup>Pflüger and Junkersdorf *Pflüger's Archiv*, cxvii, pp 201-301, 1909

<sup>2</sup>Howe, Mattill and Hawk (this *Journal*, vii, p 147, 1910) have recorded the longest fast on record, i e., 117 days. On the 117th day the dog showed a loss of 63 per cent of body weight. The animal was carefully fed and brought back to nitrogen equilibrium.

TABLE XVIII  
Dog 17—Phosphorus poisoning and starvation

DATE	BODY WEIGHT kilos	URINE					REACTION to Urinus	Total N gms	Creatinine N mgms	Creatinine V mgms	Creatinine V percent	Creatinine V percent	REMARKS
		Volume cc	Specific gravity	Reaction to Urinus	Total N gms	Creatinine N mgms							
May													
10	16 0	150	1 030	Acid	5 80	157	26	2 7	0 1				
11	16 0	125	1 038	Acid	4 92	167	20	3 4	0 1				
12	16 0	120	1 035	Acid	4 38	107	57	3 8	1 3				2 cc phosphorus oil
13	15 7	105	1 040	Acid	3 75	118	50	3 9	1 3				
14	15 5												
15	15 2	250	1 040	Acid	7 71	300	85	3 9	1 1				
16	15 2	110	1 038	Acid	3 06	150	48	3 8	1 2				2 cc phosphorus oil
17	15 0	125	1 040	Acid	3 87	113	11	3 7	1 1				
18	14 8												
19	14 2	275	1 040	Acid	10 56	375	58	3 6	0 5				2 cc phosphorus oil
20	14 0												
21	14 0	175	1 045	Acid	6 84	215	26	3 1	0 1				
22	13 9												
23	13 8	270	1 036	Acid	9 21	204	68	2 2	0 7				2 cc phosphorus oil
24	13 7												
25	13 6	190	1 042	Acid	7 91	176	31	2 2	0 1				
26	13 3	275	1 045	Acid	11 70	187	102	1 6	0 9				3 cc phosphorus oil
27		200	1 040	Acid	7 41	118	89	1 6	1 2				
28		240	1 045	Acid	10 08	135	105	1 3	1 6				
29		220	1 038	Acid	9 06	111	155	1 3	1 7				
30	12 2	175	1 045	Acid	12 21	150	250	1 2	2 0				3 cc phosphorus oil
31		300	1 040	Acid	9 60	126	271	1 3	2 9				
June													
1	11 2	360	1 032	Acid	9 84	115	271	1 2	2 8				3 cc phosphorus oil
2	210*		1 030	Acid	5 07*	65*	185*	1 3	3 6				Not complete day's urine
3	10 1	275	1 035	Acid	8 70	97	228	1 1	2 6				3 cc phosphorus oil
4	50*		1 030	Acid	0 87*	13*	59*	1 5	6 8				Not complete day's urine Animal died during night

\*Not complete day's urine, see notes

siderable amounts is of particular interest in view of the recently published paper of Frank and Isaac<sup>1</sup> These investigators found that during phosphorus poisoning, the amount of sugar in the blood practically disappears If carbohydrates are necessary for normal cellular metabolism, or for the conversion of creatine into creatinine, as has been suggested, then creatine would be expected under such conditions Lusk ('07) was unable to detect any significant change in creatinine output after phosphorus poisoning, in one experiment on a fasting dog No creatine estimations were reported Lefmann ('08) concluded that the creatinine elimination was increased during the poisoning not accompanied by fasting, while the creatine output remained unaltered His data, however, are so irregular that definite conclusions are impossible

The parallelism between *total creatinine* and total nitrogen elimination is very striking in the phosphorus-intoxicated dog as well as in the experiments previously described An increase in total creatinine is, without exception, accompanied by an increase in total nitrogen There is, however, one difference in the results obtained on dogs as contrasted with those obtained on rabbits In dogs the excretion of creatine is usually accompanied by a decrease in creatinine, though not commensurate with the increase in creatine, while in rabbits the creatinine is comparatively constant in amount, or shows only a very slight tendency to decrease during the last days of the fasting period The behavior of dogs would seem to indicate that creatine and creatinine have the same origin in the organism, and that while the production of creatine increases as starvation progresses, less and less conversion to the anhydride is accomplished

The protocols of Cathcart ('09) show a decrease in creatinine excretion in man, coincident with the increase in creatine, but contrary to the present findings no parallelism exists between the elimination of *total creatinine* and total nitrogen in his experiments Moreover, he makes no reference to the possible importance of carbohydrates in bringing about the *conversion* of creatine to creatinine

<sup>1</sup>Frank and Isaac *Arch f exp Path u Pharm*, lvi, pp 274-92, 1911

## GENERAL DISCUSSION

Two fundamental facts are emphasized by the experiments recorded (1) *An increase in the elimination of total creatinine (i e, creatine plus creatinine) is always accompanied by an increase in the output of total nitrogen, and (2) Carbohydrates, in contrast to the other food-stuffs, are capable of preventing the excretion of creatine, and are therefore indispensable for normal creatine-creatinine metabolism*

Although an increased output of nitrogen in the form of creatine and creatinine is associated with a rise in total nitrogen elimination, it by no means follows that the reverse is true. It is necessary to remember that under ordinary conditions we have in the animal body three sources of urinary nitrogen, namely food nitrogen, reserve nitrogen, and tissue nitrogen. During fasting the food nitrogen does not enter into the problem. The urinary nitrogen in inanition, therefore, has its origin in (a), reserve nitrogen (variously termed circulating protein, Vorratseiwiss, Reserveeiweiss, Zelleneinschluss, labiles Eiweiss, by different writers), and (b) disintegration of organized body tissue—so-called endogenous nitrogenous metabolism. If the former were its source, no accompanying increase in total creatinine should occur, for it is probable that this form of nitrogen is metabolized just as the (exogenous) food nitrogen. When, on the other hand, it becomes necessary for the tissues to disintegrate to furnish energy for the organism, an increase in creatine or creatinine necessarily occurs. The most abundant tissue, and one which suffers great loss in weight, namely, muscle tissue, contains considerable creatine. This will be liberated and might be expected to appear in the urine either unaltered or as creatinine.

But this is not the only factor involved. Considerable evidence has been accumulated in recent years, indicating that creatine is a product of endogenous metabolism, and that an increased formation of creatine occurs when the tissue catabolic processes are accelerated. There is evidence, also, that creatine can in part disappear in the body, and in part be converted into creatinine. Of importance in this connection, is the work of Gottlieb and his collaborators. Gottlieb and Stangassinger ('07, '08), and Stangassinger ('08), found that during the autolysis of muscle and other

organs, a formation of creatine occurs. The creatine so arising, as well as creatine added to the autolytic mixture is, by the action of an enzyme, partially converted into creatinine. Both creatine and creatinine are by long continued autolysis, destroyed through the action of specific enzymes (creatase and creatinase). The very complex curves representing the amounts of creatine and creatinine in an autolytic mixture depend, therefore, upon the balance between formation, conversion, and destruction of these substances.

The results of Gottlieb's experiments have been severely criticised by Mellanby ('08), who concluded that when the autolytic experiments were kept rigorously free from bacteria, and when precautions were taken to prevent the conversion of creatine into creatinine by heating, no change occurred during autolysis. The experiments have, however, been repeated with improved technic by Rothmann ('08) and van Hoogenhuyze and Verploegh ('08), and results obtained similar to those of Gottlieb.

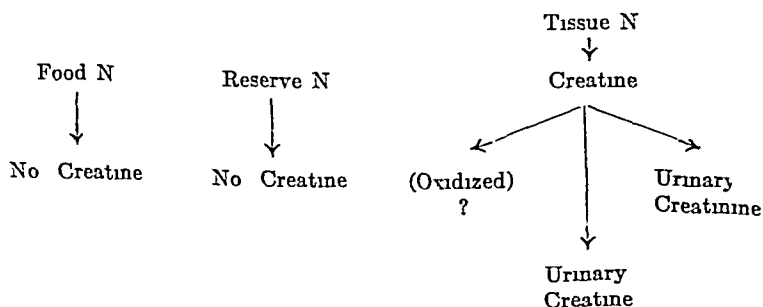
Whether one accepts Gottlieb's or Mellanby's experiments, the fact remains that in muscle tissue an increased production of creatine may actually occur under appropriate conditions. Weber ('07) and Howell and Duke ('08) found that the beating heart liberates creatine into the perfusion fluid. Weber also observed that the creatinine excretion is considerably higher in dogs poisoned with cinchonine, indicating that increased tonus may lead to creatine or creatinine formation. Likewise Graham-Brown and Cathcart ('08) found that stimulation of isolated frog muscles brought about an increase of from 7 to 13 per cent in the creatine content. Van Hoogenhuyze and Verploegh ('08) found the excretion of creatinine to be greater during the day, when the muscle wear and tear is increased, than during the night when the muscle tonus is reduced.

More recently Pekelharing and van Hoogenhuyze ('10a) have demonstrated an increased formation of creatine in the muscle during *rigor caloris*, *rigor mortis*, and heightened tonus. These authors believe that during tonus muscle protein is decomposed to furnish energy, and that creatine is a product of this endogenous catabolism. The sources of the energy utilized for the maintenance of tonus and for the performance of ordinary muscular work are, according to this view, entirely different. If this theory is correct, an increased formation of creatine should occur during fasting.

after the supply of non-nitrogenous energy-yielding food-stuffs and of nitrogenous reserve material has been depleted, and should not occur until such a depletion has been effected. The theory and the facts obtained in the experiments already described are, therefore, entirely in accord.

The numerous other observations of creatine excretion in conditions associated with wasting of muscle tissue, as in fevers (Shaffer, '08b, and van Hoogenhuyze and Verploegh, '08), during the *post partum* resolution of the uterus (Shaffer, '08b, and Murlin, '08-'09), and in muscular disease (Levene and Kristeller, '09), all point to the same conclusion, namely, that whenever muscle protein is decomposed, creatine is a product of the disintegration. Further evidence of this will be found in a marked increase of the creatine content of muscle during starvation, in hens and rabbits.<sup>1</sup>

At the present time the most probable explanation of the production of creatine and creatinine may be sought in the catabolism of the tissues (*i e*, endogenous metabolism). Under appropriate nutritive conditions, the small amount of creatine arising from muscle wear and tear, is converted into creatinine and excreted. When, however, an undue creatine production occurs, the conversion to creatinine may become inadequate, and creatine as such appear in the urine. Possibly some may be oxidized and not appear at all. In this case creatine and creatinine would be analogous to uric acid and represent a balance between formation and destruction. They would then be intermediary rather than end products. These views are represented in the accompanying scheme.



<sup>1</sup>The experimental proof of this will be furnished in the next paper



It may be objected that if this theory were true creatine introduced *per os* or parenterally should ordinarily be converted into creatinine, and that this is contrary to the observations of most investigators (Folin, '06, Klercker, '06 and '07, Lefmann, '08). But this does not follow any more than that creatine arising during starvation should be converted into creatinine. It is possible that the organism is capable of converting only a definite amount of creatine into the anhydride, and that when this amount is exceeded, unaltered creatine appears in the urine. On the other hand, a slight conversion was observed by van Hoogenhuyze and Verploegh ('08). In the most recent paper along this line, Pekelharing and van Hoogenhuyze ('10b) found that creatine parenterally introduced into rabbits and dogs, was partly transformed into creatinine, partly oxidized, and partly excreted unaltered. It must be remembered also that in injection experiments the circulation may be so flooded with creatine, that there is not sufficient time for conversion before elimination occurs. In many of the earlier experiments, where creatine was given by mouth, it is not improbable that it was largely decomposed by bacteria in the alimentary canal (cf Czernecki, '05, and Nawiasky, '08). Plimmer, Dick and Lieb ('09-'10), in experiments in man, found that 2.5 grams of creatine had to be given by mouth before any could be recovered in the urine. This may serve to explain the entire disappearance of creatine in many feeding experiments, and its failure to increase the creatinine output.

With our present knowledge, it is impossible to formulate anything definite as to the chemical processes by which creatine arises in tissue catabolism. The striking similarity between its structure and the structures of many other substances occurring in muscle tissue, or derived from proteins, indicates that its origin in tissue catabolism is by no means inconceivable. Attempts, however, to associate these compounds with creatine and creatinine experimentally, have thus far been unsuccessful or doubtful (cf Burton, '05, Jaffé, '06, Achels, '06, Dorner, '07, and Lefmann, '08).

It is difficult to form any chemical picture of the influence carbohydrates may have in preventing the excretion of creatine. As already suggested, they may be necessary for the conversion of creatine into creatinine, or in their presence creatine may be more readily oxidized and excreted as urea. Again, the tissue cells

may not functionate properly when the normal amount of carbohydrate food is wanting, and in this case the elimination of creatine would be analogous to the production of the acetone bodies, which is also inhibited by the administration of carbohydrates. More work will be necessary to elucidate these problems. *Without question the metabolism of creatine is intimately associated with carbohydrate metabolism*

#### SUMMARY

1 The excretion of creatine induced by starvation, is inhibited in rabbits by feeding a diet of carbohydrates absolutely free from proteins and fats. When the carbohydrates are given in liberal amounts, creatine entirely disappears from the urine.

2 The creatine elimination is not reduced by feeding a diet of fat alone, or by a diet of fat and protein.

3 Experimental interference with carbohydrate metabolism leads to the elimination of creatine. After phlorhizin diabetes which depletes the store of carbohydrates, and during phosphorus poisoning, which disturbs the glycogenic functions, the output of creatine in dogs is decidedly increased.

4 An increase in the output of creatine plus creatinine (total creatinine), is always accompanied by an increase in total nitrogen elimination. This parallelism of total creatinine and total nitrogen outputs in manition and with nitrogen-free diets is ascribed to a common source, namely, true tissue or endogenous metabolism. The metabolism of exogenous or reserve proteins is not accompanied by the production of creatine or creatinine.

5 The intimate relation of creatine excretion (or the failure of conversion into creatinine) to carbohydrate metabolism, is discussed in detail.

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## EXPERIMENTAL STUDIES ON CREATINE AND CREATININE

### II INANITION AND THE CREATINE CONTENT OF MUSCLE<sup>1</sup>

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(Received for publication, August 14, 1911)

Comparatively few investigations have been made in regard to the creatine content of muscle in normal and abnormal conditions. Such data as we have were, for the most part, obtained with the older and inadequate analytical method of Neubauer and are consequently usually unreliable. Many of these researches were undertaken with a view of determining the influence of muscular work on the content of creatine, but because of the errors in technic the results have been widely different.

Liebig ('47) was the first to begin systematic studies on the effect of activity on tissue composition. In 1847 he made his classical investigation of the creatine content of muscle in fatigue and found that the muscle of a fox killed in the chase contained ten times the amount of creatine present in an equal weight of muscle from a resting animal. Sarokow ('63) also found an increase of creatine after work. He believed that coincident with the formation of creatine, the muscular activity brought about a partial conversion into creatinine. A similar increase in creatine was reported by Sczelkow ('66) as the result of tetanus, while rest was supposed to produce a decrease. On the other hand Nawrocki ('66) in experiments on dogs, found no difference in the creatine content of tetanized and resting muscle.

The results of Voit ('68) were again different. In experiments on frogs, he decided that tetanus produced a decrease in the

<sup>1</sup> The experimental data are taken from the thesis presented by William C. Rose for the degree of Doctor of Philosophy, Yale University, 1911.

creatine content of muscle According to this investigator the creatine is transformed during work to a substance not identical with creatinine

The most consistent of the earlier results on the effect of work were obtained by Monari ('87) who found that an increase in creatine invariably occurred in fatigue, and that coincident with the increase a much larger amount of creatinine was detectable The protocols of Monari appear very conclusive, and were accepted and frequently quoted in subsequent papers It seems probable now from the investigations of Mellanby ('07-'08), that much of the precipitate which Monari weighed as pure creatine, was composed of other substances The creatinine obtained by him was undoubtedly derived from creatine in the evaporation of the acid tissue extracts That preformed creatinine does not exist in freshly killed muscle is definitely proven by the researches of Grindley and Woods ('06-'07), Mellanby ('07-'08), and Laton ('09-'10)

Since the introduction of the Folin colorimetric method for the estimation of creatinine, several papers have appeared on the influence of activity on muscle creatine Weber ('07) found that work caused a slight decrease in the creatine content, and that the beating heart gave off creatine or creatinine into the perfusion fluid According to this author, less creatine was present in degenerated than in normal muscle Mellanby ('07-'08), on the other hand, found that the performance of muscular work, as well as the survival of isolated muscle, leaves creatine unaffected No change in the creatine content was found by von Furth and Schwarz ('11) after tetanizing the hind-leg muscles of a dog for more than an hour

Graham-Brown and Cathcart ('08) report that stimulation produces an increase in the amount of total creatinine in isolated frog muscles, whereas it leads to a slight decrease when the circulation is left intact In a later communication, these authors (Graham-Brown and Cathcart, '09) report the results of studies made on rabbits, in which they find that with the circulation intact, stimulation induces a constant though small decrease in the amount of total creatinine (*i e*, creatine plus creatinine)

Practically no data have been published on the effect of factors other than activity on the creatine content of muscles By the

use of the Neubauer method Demant ('79) observed an increase in the percentage of creatine in the breast muscles of pigeons during starvation. All figures given by this author are much lower than those obtained by the use of the Folin method. From the analysis of the muscle of a single starving rabbit Dorner ('07, p 261), using the Folin method, concluded that there was a decrease in creatine during inanition. Recently, in a preliminary report, Howe and Hawk ('11) claim that a very marked reduction in the creatine of dog's muscles is brought about by fasting.

Such studies are of prime importance in determining the origin of urinary creatine and creatinine. If it is universally true that a decrease in the creatine content of muscle occurs during inanition, the creatine found in the urine during starvation must have its origin in a "washing-out" of muscle creatine. If, on the other hand, the accelerated endogenous metabolism during starvation occasions an increased formation of creatine, as has been suggested in a previous paper (cf Mendel and Rose '11), then urinary creatine and creatinine would represent local metabolic end-products, rather than substances merely washed out of the tissues. The excess of creatine produced during such a process might be entirely excreted or oxidized, or partly retained in the muscle. The muscle analyses would then show either no change or an increase in the creatine content, but never a decrease.

To determine this question a series of analyses was made of muscle tissue removed from normal and starving rabbits and hens, the results of which are summarized in Tables II and III. The animals were allowed to starve for varying lengths of time and killed by bleeding. To avoid errors which might arise from differences in the amount of creatine in different muscles, similar muscles were always selected in the control and experimental animals. In the rabbits, the muscle tissue from the hind legs and back was completely removed, freed as much as possible from connective tissue, and thoroughly ground in a hashing machine. From the uniform mixture samples were rapidly weighed for the analyses. In the fowl, only the pectoral muscles were used.

The total creatinine was estimated according to the procedure of Mellanby.<sup>1</sup>

<sup>1</sup>Mellanby *Journ of Physiol*, xxvi, pp 453-4, 1907-8



For this purpose, the finely ground muscle was killed by covering with 95 per cent alcohol. The alcohol was poured off through silk gauze, the muscle pressed out, and repeatedly extracted by shaking with five portions of water, about half an hour being allowed for the extraction with each portion. The alcoholic and watery extracts were then combined and evaporated to dryness on the water-bath. The residue was extracted five times with 75 per cent alcohol, which removed the creatine and creatinine, but left most of the protein behind. The alcohol was removed by evaporation, the solution made up to a known volume with water—usually 150 cc—filtered, and the total creatinine determined on 10 cc portions by the Folin-Benedict-Myers method. Preliminary analyses showed that very good duplicates could be obtained by this procedure.

In the rabbits, simultaneous determinations were made of the water, ether-extract, and ash of the muscles. Since only slight variations in the percentages of ether-extract and ash occurred, these estimations were omitted in the fowl. In the rabbits the creatine content is calculated on the moist muscle, and on the ether-extract- and fat-free dry material, while in the fowl the percentages are calculated on the bases of moist and dry tissue.

The amount of *water* in the normal tissues is reasonably constant. The higher figure obtained with rabbit 4 is due to the fact that the animal had received an intravenous injection of 150 cc of dilute adrenalin solution, in connection with other investigations. Only a small amount of the injected water had been excreted when death occurred. In the starving animals, the water content progressively increased as starvation was prolonged. Similar results were obtained by v. Boethlingk<sup>1</sup> on mice. This increase in water may have caused the apparent decrease in the amount of creatine observed by Dorner, and calculated by him on the moist material.

The *ether-extract* is also constant in the normal animals, and shows a decrease during fasting. The figures agree with those found in starving rabbits by Rubner<sup>2</sup>. This author reports that in starvation the muscles of rabbits contain 2 to 3 per cent of fat calculated on the dry material. Assuming that the muscles of his animals contained approximately 80 per cent of water, his figures agree with those reported in Table II. The percentage of fat in the well-fed rabbits is somewhat lower than that reported

<sup>1</sup>v. Boethlingk. *Arch d scienc biol*, v, p 395, 1897

<sup>2</sup>Rubner. *Zeitschr f Biol*, xvii, p 229, 1881

for the wild hare by König and Farwick,<sup>1</sup> who found 1.07 per cent of ether-extract in the muscles of the extremities. Moreover, the fat content of other animals is usually higher than that of the rabbit. According to the analyses of Almén,<sup>2</sup> the muscles of lean oxen contain 1.5 per cent of fat and 76.7 per cent of water, while the muscles of pigeons contain 1.0 per cent of fat.<sup>3</sup> This is in accord with the well known fact that rabbits usually have relatively little subcutaneous adipose tissue. They seem to store fat only with difficulty.

TABLE I  
*The creatine content of muscle*

AUTHOR	REFERENCE	ANIMAL	CONDITION OF MUSCLE	CREATINE IN MOIST TISSUE
Mellanby	Journ. Physiol., xxxvi, p 472, 1907-8	Frog	Normal	per cent 0.302
Graham- Brown and Cathcart	Biochem. Journ., iv, p 421, 1909	Frog	Normal	0.377
		Frog	Isolated and stim.	0.413
Mellanby	Journ. Physiol., xxxvi, p 472, 1907-8	Fowl	Normal	0.360
Mellanby	Journ. Physiol., xxxvi, p 460, 1907-8	Rabbit	Normal (leg)	0.520
Mellanby	Journ. Physiol., xxxiv, p 460, 1907-8	Rabbit	Normal (back)	0.505
Mellanby	Journ. Physiol., xxxiv, p 460, 1907-8	Rabbit	Stimulated	0.506
Dorner	Zeitschr. f. physiol. Ch., lii, p 265, 1907	Rabbit	Normal	0.529
Dorner	Zeitschr. f. physiol. Ch., lii, p 265, 1907	Rabbit	Normal	0.496
Dorner	Zeitschr. f. physiol. Ch., lii, p 265, 1907	Rabbit	Normal	0.496
Dorner	Zeitschr. f. physiol. Ch., lii, p 265, 1907	Rabbit	Normal	0.505
Dorner	Zeitschr. f. physiol. Ch., lii, p 265, 1907	Rabbit	Starving	0.414

<sup>1</sup>König and Farwick. *Zeitschr. f. Biol.*, xii, p 497, 1876

<sup>2</sup>Almén. *Nora Act. Reg. Soc. Scient. Upsal*, vol. extr. ord., 1877

<sup>3</sup>Cf König. *Chem. d. menschl. Nahrungs- u. Genussmittel*, i, p 42, 1903

TABLE II  
*Creatine in starving rabbits' muscle*

NUMBER OF ANIMAL	DURATION OF STARVATION	INITIAL WEIGHT	FINAL WEIGHT	LOSS IN WEIGHT	LOSS IN WEIGHT	MUSCLE ANALYSES						Creatine in water-, ether-, and ash-free muscle
						Water	Ether extract	Ash	Muscle used for creatine estimated	Creatine recovered	Creatine in moist muscle	
	days	grams	grams	percent	percent	percent	percent	percent	grams	gram	percent	percent
1	0	2260	2260	0	0	76 20	0 72	1 16	41 87	0 188	0 449	2 05
2	0	2600	2600	0	0	75 20	0 61	1 19	40 50	0 196	0 484	2 11
3	0	2440	2440	0	0	74 87	0 77	1 21	40 89	0 216	0 528	2 29
4*	0	2360	2360	0	0	77 28*	0 41	1 18	41 00	0 218	0 532	2 52
5	7½	2220	1660	560	25 2	77 42	0 39	1 17	41 90	0 224	0 535	2 54
6	10½	1900	1420	480	25 3	77 58	0 58	1 21	41 50	0 251	0 605	2 62
7	16	2380	1610	770	32 4	78 57	0 44	1 10	41 96	0 238	0 567	2 85
8	14	2320	1480	840	36 2	78 38	0 43	1 14	40 66	0 257	0 632	3 15
9	13	2140	1320	820	38 3	80 76	0 53	1 10	40 55	0 238	0 587	3 33
10	20	2400	1390	1010	42 1	77 76	0 40	1 16	41 20	0 244	0 592	2 86
11	9	2140	1210	930	43 5	80 69	0 41	1 14	36 55	0 213	0 583	3 28
12	13	2540	1410	1130	44 5	79 48	0 43	1 15	40 78	0 190	0 466	2 46
13	20	2200	1120	1080	49 1	79 99	0 33	1 02	40 93	0 235	0 574	3 08

\*Animal died during the injection of dilute adrenalin solution in connection with other investigations. This accounts for the high water content of the muscle.

The amount of *ash* undergoes very little change in rabbits during fasting. The figures compare favorably with those found by König and Krauch,<sup>1</sup> the average of which was 1.17 per cent.

**Creatine content.** A summary of the more important records of the creatine content of muscle is given in Table I. Where these figures were expressed as creatinine in the original papers, they have been recalculated by us and expressed as creatine. A comparison of these data with our analyses will show a good agreement in the creatine content of muscle in so far as normal, *i.e.*, fed animals are concerned.

TABLE III  
*Creatine in starving hens' muscle*

NUMBER OF ANIMAL	DURATION OF STARVATION	INITIAL WEIGHT	FINAL WEIGHT	LOSS IN WEIGHT	LOSS IN WEIGHT	MUSCLE ANALYSES					
						Water	Muscle used for creatine estimated	( creatine recovered	Creatine in moist muscle	Creatine in dry muscle	
	days	grams	grams	grams	percent	percent	grams	gram	percent	percent	
14	0	1750	1750	0	0	73 00	41 34	0 170	0 411	1 52	
15	0	1890	1890	0	0	73 05	41 08	0 170	0 414	1 54	
16	11	2160	1775	385	17 8	73 31	41 25	0 181	0 439	1 64	
17	19	2260	1845	415	18 4	73 66	40 40	0 200	0 495	1 88	
18	11	2160	1710	450	20 8	73 24	41 45	0 184	0 444	1 66	
19	17	1170	820	350	30 0	76 27	22 61	0 087	0 384	1 62	
20	19	1520	1000	520	34 2	76 85	32 12	0 138	0 430	1 85	

The data obtained in the present investigation of starving animals, on the other hand, exhibit distinctly higher values. There are few comparable experiments on record. The protocols are arranged in the Tables (II and III) in the order of the progressive percentage loss in weight of the animals. With a single exception (rabbit 12) the muscles of all starving animals showed a higher percentage of creatine than the fed controls. The increase in creatine tends to be proportional to the percentage loss in weight of the animals. No explanation can be given for the isolated low figures obtained with animal 12. The difference may have been due in part to age, for Mellanby ('07-'08) has shown that considerable variation in the creatine content is associated with this factor. Young animals have much less creatine than adults.

<sup>1</sup> König and Krauch. *Chem. d. menschl. Nahrung u. Genussmittel*, 1, p. 40, 1903.

In the experiments with *hens* the results are comparable to those obtained on rabbits. Both water and creatine increased in percentage as starvation was prolonged. In fowl 19 the creatine calculated on the moist muscle is lower than in the control birds, but when expressed on the basis of the dry tissue, is slightly higher than the controls. The low figures here are without doubt due to age, for this fowl was small and immature.

It is noticeable that the increases in creatine in the fowl are not as large as in the rabbits. Most of the former were in good nutritive condition when killed. All but the half-grown hen (No. 19) had considerable subcutaneous fat. Since they retain fat so readily, it may be equally true that they retain considerable glycogen, and a marked increase in creatine would not be expected. It is certainly true that as a rule fowl can fast much longer than rabbits before death results.

#### DISCUSSION

Since a significant and progressive increase in creatine has been demonstrated in muscle during inanition, there seems to be no reasonable ground for doubting the origin of urinary creatine and creatinine in endogenous metabolism. These results are in striking contrast to those reported for a dog by Howe and Hawk ('11). They (*loc. cit.*, p. 239) estimated that "the amount of creatine nitrogen present in the muscles of the dog at the end of the fast was 0.042 per cent, showing a very marked decrease (66 per cent)." It is not apparent in the preliminary report of Howe and Hawk whether this finding was duplicated or not. In order to prove conclusively such a remarkable reduction, it would be necessary to have data on a number of animals of approximately the same age as the controls.

Howe and Hawk believe that the pronounced decrease in creatine is "a most significant fact and shows clearly that in fasting we cannot with accuracy consider the total amount of excreted creatine as resulting from the complete and permanent disintegration of muscular tissue." Again they say, "a large part of the creatine excreted during the fasts and which is ordinarily considered as representing completely disintegrated muscular tissue, in reality most certainly does not represent this but rather has been withdrawn from muscular tissue which is still functioning as living tissue within the body of the animal." It is difficult to under-

stand how any such withdrawal of creatine from functioning tissue could occur. Urino ('07) has shown that muscle creatine is held in a non-diffusible form and is probably loosely combined with the muscle protoplasm. Its liberation would only be possible after complete disintegration of the muscle bundles.

As evidence that creatine is not an index of muscle disintegration, Howe and Hawk point to the discrepancy between the amounts of muscle catabolism calculated on the two bases of total nitrogen and creatine excretion. They find that only about one-half of the urinary nitrogen can be accounted for, when the tissue catabolism is calculated on the creatine basis. Several sources of error are apparent in such a calculation. The authors seem to assume that all urinary nitrogen has its origin in muscle catabolism. It is true that muscle represents the major part of the body tissues, and that it undergoes a great loss in weight during inanition, but Voit<sup>1</sup> has shown that the glandular tissue undergoes much greater loss in weight than does muscle. Creatine may be a product of the metabolism of glandular tissue, but with our present knowledge there is certainly no evidence for such an assumption. Moreover, dogs invariably have an enormous store of reserve nitrogen, which might increase the output of total nitrogen during starvation without altering the creatine excretion. But the greatest fallacy in the reasoning of Howe and Hawk is that they entirely neglect to consider the output of preformed creatinine in their calculations, and apparently assume for it an origin distinct from that of creatine. If the tissue catabolism is calculated on the basis of the output of total creatinine (*i e*, creatine plus creatinine), the discrepancy between the figure so obtained, and that obtained on the total nitrogen basis, will be found to be much smaller. Indeed, in the second fasting period of Howe and Hawk's dog, the muscle waste calculated from the total creatinine, is more than sufficient to account for all urinary nitrogen.

All methods of calculating tissue loss on this basis must be inaccurate. The catabolism of tissues other than muscle tissue, the excretion of reserve nitrogen, the possibility of a resynthesis of nitrogen (*cf* Paton, '09-'10), changes in the rate of formation, oxidation, retention, and excretion of creatine and creatinine, all tend to render such calculation inconclusive. The fact that

<sup>1</sup>Voit *Hermann's Handbuch*, vi, part 1, pp 96-97

calculations dependent upon so many variables do not exactly agree, is not surprising. It would rather be surprising if they did agree. Hence, by these methods no evidence can be obtained which would invalidate the hypothesis that creatine and creatinine are products of the endogenous metabolism of muscle tissue.

Without doubt there occurs an increase in the percentage of creatine in the muscles of rabbits and fowl during inanition. Two possible explanations for the larger amount of creatine suggest themselves. The increase may be due (1) to a removal of the non-creatine portion of the muscle, leaving the creatine intact, or (2) to an increased formation of creatine. The first explanation seems improbable, for if the creatine is loosely combined with muscle as Urano ('07) believes, there is no reason for assuming that the non-creatine material could be withdrawn and leave the creatine intact, any more than for assuming that the reverse could occur. An increased formation seems to be the most plausible explanation. This view is in accord with the numerous observations already cited in a former paper (Mendel and Rose, '11). During such an accelerated production of creatine, it would probably be liberated in part by the muscles and appear in the urine, for the muscles would become supersaturated and be unable to combine with all of the excess.

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# EXPERIMENTAL STUDIES ON CREATINE AND CREATININE

## III EXCRETION OF CREATINE IN INFANCY AND CHILDHOOD<sup>1</sup>

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(Received for publication, August 14, 1911)

Physiological literature contains scarcely any data on the creatine-creatinine metabolism of children. Before the introduction of the Folin method for the estimation of these substances, creatinine was supposed to be entirely absent from the urine of the young. Riettschel ('05) was unable to detect it in normal suckling infants by the Salkowski-Neubauer method, or by the color reaction of Weyl. Van Hoogenhuyze and Verploegh ('05), Amberg and Morrill ('07), Funaro ('08), Amberg and Rowntree ('10), and Sedgwick ('10), using the Folin method, have now conclusively demonstrated that creatinine is a constant constituent of the urine of sucklings. All investigators find the creatinine excretion to be very low, ranging usually from 6 to 10 mgms per kilo of body weight.

Assuming the origin of urinary creatine and creatinine to be muscle creatine, one would expect to find creatinine in the urine of sucklings. Mendel and Leavenworth ('08) have shown that the embryonic muscles of the pig contain an average creatine content of 0.03 per cent of the moist tissue. An analysis made by the writer of the muscles of a new born infant (see Table I) indicates that creatine is present in somewhat larger amount than in embryonic muscle, though still far less than the amount present in the muscles of an adult. These observations are in accord with those of Mel-

<sup>1</sup>The data are taken from the thesis presented by the author for the degree of Doctor of Philosophy, Yale University, 1911.



lanby ('08)—that the creatine content of muscle greatly varies with the age of the animal. They serve also to throw some light on the extremely low creatinine-coefficients of sucklings, and furnish further evidence for the origin of urinary creatinine in muscle metabolism.

TABLE I

*Analysis of the muscle of a new-born infant*

WATER	ASH	ETHER EXTRACT	MUSCLE USED FOR CREATINE ESTIMATION	CREATINE FOUND	CREATINE IN MOIST MUSCLE	CREATINE IN WATER, ASH, AND ETHER EXTRACT FREE MUSCLE
<i>per cent</i> 80 12	<i>per cent</i> 0 69	<i>per cent</i> 5 94	<i>grams</i> 37 03	<i>gram</i> 0 072	<i>per cent</i> 0 19	<i>per cent</i> 1 46

Amberg and Morrill ('07) found creatine to be present in the urine of a single infant tested by them. Similar observations were made on sucklings by Sedgwick ('10) and van Hoogenhuyze and ten Doeschate ('11), and on young puppies and kittens by Closson ('06). In one experiment on a puppy three weeks old, Closson found 18 mgms of total creatinine to be excreted per day, of which 17 mgms were in the form of creatine. At the age of eight and a half weeks, the total creatinine excretion per day was 48 mgms, of which 29 mgms were in the form of creatine.

The data regarding the elimination of creatine in children more than a year of age, are exceedingly scanty. Schwarz ('10) reported that a normal five-year-old boy examined by him excreted no creatine, while rachitic children of the same age excreted 30 to 50 per cent of their total creatinine in the form of creatine.

In view of the observations that creatine is a normal constituent of the urine of sucklings, it was of interest to determine the age at which this product disappeared. Numerous specimens of urine from children of different ages were analyzed, the result of which are summarized in Tables II and III. Since it was impractical to collect the complete excretion for twenty-four hour periods, the analytical figures are expressed in milligrams per one hundred cubic centimeters of urine. Specimen 24 was obtained from a child convalescing after an attack of mumps, all other samples were obtained from apparently perfectly normal individuals.

TABLE II

*Creatine in the urine of male children    Amount in 100 cc*

NUMBER OF SAMPLE	AGE OF CHILD	URINE						REMARKS
		Specific Gravity	Reaction to litmus	Total creatinine	Preformed creatinine	Creatine as creatinine	Creatine in per cent of total creatinine	
1	years 1½	1 030	Acid	mgms 110	mgms 88	mgms 22	20 0	Perfectly normal
2	4	1 021	Acid	66	47	19	28 8	Perfectly normal
3	5	1 025	Acid	66	50	16	24 2	Perfectly normal
4	5	1 017	Acid	159	33	126	79 2	Sample from same child as No 3
5	10	1 025	Acid	92	75	17	18 5	Perfectly normal
6	10		Acid	75	60	15	20 0	Perfectly normal
7	10	1 020	Acid	70	70	0	0	Perfectly normal
8	11	1 023	Acid	113	96	17	15 0	Perfectly normal
9	11	1 022	Acid	135	116	19	14 1	Protein present
10	12	1 025	Acid	156	100	56	35 9	Perfectly normal
11	13	1 019	Acid	81	74	7	8 6	Perfectly normal
12	14	1 020	Acid	68	49	19	27 9	Perfectly normal
13	15		Acid	87	65	22	25 3	Perfectly normal
14	17	1 022	Acid	133	133	0	0	Perfectly normal
15	18	1 028	Acid	121	121	0	0	Perfectly normal
16	18	1 030	Acid	270	270	0	0	Perfectly normal
17	18	1 028	Acid	175	175	0	0	Perfectly normal
18	19	1 024	Acid	144	144	0	0	Perfectly normal
19	19	1 018	Acid	112	112	0	0	Perfectly normal

TABLE III

*Creatine in the urine of female children Amount in 100 cc*

NUMBER OF SAMPLE	AGE OF CHILD	URINE						REMARKS
		Specific gravity	Reaction to litmus	Total creatinine	Preformed creatinine	Creatine as creatinine	Creatine in per cent of total creatinine	
	<i>years</i>			<i>mgms</i>	<i>mgms</i>	<i>mgms</i>		
20	1½	1 010	Acid	16	14	2	12 5	Perfectly normal child
21	1½	1 005	Acid	17	7	10	58 8	Perfectly normal child
22	3	1 030	Neutral	53	38	15	28 3	Perfectly normal child
23	3	1 018	Acid	68	40	28	41 2	Perfectly normal child
24	5	1 030	Alkaline	72	60	12	16 7	Mumps
25	7	1 019	Acid	61	27	34	55 7	Perfectly normal child
26	7	1 020	Acid	66	50	16	24 2	Sample from same child as No 25
27	7	1 024	Alkaline	60	53	7	11 7	Perfectly normal child
28	8	1 021	Acid	41	34	7	17 1	Perfectly normal child
29	8	1 030	Acid	90	58	32	35 6	Sample from same child as No 28
30	11	1 913	Acid	64	52	12	18 8	Perfectly normal child
31	11	1 020	Acid	79	69	10	12 7	Perfectly normal child
32	12	1 020	Acid	91	62	29	31 9	Minute trace of protein
33	13	1 025	Acid	91	91	0	0	Perfectly normal child
34	13	1 016	Acid	60	49	11	18 3	Perfectly normal child
35	13	1 022	Acid	117	86	31	26 5	Perfectly normal child
36	15	1 023	Acid	83	61	22	26 5	Trace of protein present
37	15	1 016	Acid	72	60	12	16 7	Same as 36 Protein present
38	20	1 014	Acid	69	69	0	0	Normal
39	21	1 016	Acid	81	81	0	0	Normal

The urines were preserved with toluene, and analyzed within twenty-four hours after excretion. Except in the cases indicated (Nos 9, 32, 36 and 37), protein and sugar were entirely absent.

Contrary to the findings of Schwarz, children of five years and over excrete considerable creatine. Indeed, with the exception of two cases, creatine was present in all specimens from children under fifteen years of age. A boy of ten and a girl of thirteen failed to have creatine in their urines.

No progressive decrease in the percentage of the total creatinine in the form of creatine coincident with increase in age is apparent, nor is the percentage of creatine constant for the same individual. For instance, one specimen (No 3) obtained from a child of five years contained 24.2 per cent of the total creatinine in the form of creatine, while a second sample (No 4) from the same child a few days later, contained 79.2 per cent of the total creatinine as creatine.

It was impossible to obtain information as to the amount and kind of food eaten by the children. Most of the specimens were obtained from the city orphans home or from private families, and the subjects of the experiments were probably ingesting more or less meat. It is possible, therefore, that the oxidation or conversion of creatine into creatinine may be difficult for young individuals to accomplish, and in this case the creatine of the urine may, in part, represent ingested creatine, or the glycogenic functions may be imperfectly developed, and the store of carbohydrates be insufficient to exert its regulatory influence over metabolism during childhood. Frank<sup>1</sup> has shown that the percentage of sugar in the blood of infants is greater than that of adults. It is conceivable that the demand for carbohydrates for the histogenetic processes may be so great that the cells are left in partial carbohydrate hunger, and are unable to perform the "endo-catabolic" activities as perfectly as in later life. At any rate it is of some interest statistically to find that creatine is usually present in the urine until or after the age of puberty.

<sup>1</sup>Frank. *Zeitschr f physiol Chem*, lxx, pp 129-42, 1910

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# STUDIES IN CARBOHYDRATE METABOLISM

## II THE PREVENTION AND INHIBITION OF PANCREATIC DIABETES

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The literature<sup>1</sup> relative to the production of pancreatic diabetes in dogs is so well-known among physiologists that a detailed recital of the conditions attending the induction of the above mentioned pathological state is unnecessary. Nevertheless, for the full appreciation of the import of the present communication it may not be out of place to call attention briefly to a few of the most salient features connected with the production of pancreatic diabetes. For temperamental and anatomical reasons the dog is best suited for this type of experimentation. With rabbits the production of pancreatic diabetes may not be constantly successful, owing to the diffuse distribution of the pancreas through the mesentery of this animal.

Accepting then as typical the results obtained with the dog, one may reasonably assume certain facts as fundamental for the purpose of further investigation. Thus, it is a matter of universal experience that glycosuria is always provoked by the practically complete removal of the pancreas in dogs whether the animals are maintained in a well-fed or fasting condition. The time of the first appearance of sugar in the urine subsequent to the operation in question is not usually specifically stated in the literature incident to such experimentation, hence such data have been supplied in the present paper under conditions strictly comparable to those found to be essential for the purposes aimed at. Another feature which may be assumed to be a constant factor in pancre-

<sup>1</sup>cf Biedl *Innere Secretionen*, Berlin, 1910

atic diabetes is the accompanying hyperglycaemia. Finally, the assumption may be made, without meriting such universal acceptance, that pancreatic diabetes is either identical with or closely akin to the pathological conditions existing in the diabetes of man.

Leaving out of consideration the decision as to the *identity* of experimental pancreatic diabetes with the human abnormal state one may reasonably assume an exceedingly close relationship between the two conditions. Any procedures, therefore, which will aid in the better understanding of the experimental diabetes, or in the prevention, inhibition or alleviation of the symptoms, are likely to have an important bearing upon our conception and treatment of the human type of pathological carbohydrate metabolism.

In a previous paper<sup>1</sup> it has been pointed out that the diamine, hydrazine, is capable of inducing an appreciable hypoglycaemia in dogs. The relation of the action of hydrazine to pancreatic diabetes forms the basis of the present communication.

#### EXPERIMENTAL

**METHODS** Unless specifically stated otherwise all operations were carried out under ether anaesthesia only. Aseptic precautions were taken throughout the operative technique. After operation the animals were carefully cared for, attention being paid to assure proper temperature of the animal room. After death of the animals operated upon for removal of the pancreas, autopsy revealed the practically entire absence of pancreas in every instance. Analysis of the blood sugar was made according to the method usually employed in this laboratory.<sup>2</sup> Sugar in the urine was estimated with a Schmidt and Haensch triple shadow saccharimeter.

#### *Experiments to determine the first appearance of sugar in the urine of dogs subsequent to removal of the pancreas*

In our previous paper intimation has been made that hydrazine is fatally toxic in large doses but that relatively small quantities

<sup>1</sup>Underhill *This Journal*, x, p. 159, 1911.

<sup>2</sup>Underhill *loc cit*

of the drug exert only a temporary detrimental influence. When the dog is subjected to a combination of hydrazine poisoning and pancreas removal death follows within a comparatively few hours. Therefore in order to determine correctly the action of hydrazine upon the course of events following pancreas removal it is imperative to know the behavior of the criterion adopted, *i e*, presence of sugar in the urine, in animals deprived of the pancreas but without the administration of hydrazine.

Two such observations have been made upon animals kept under conditions in every way identical with those depancreatized dogs subjected to the influence of hydrazine to be reported below.

EXPERIMENT 1 From a full-grown bitch of 5 kilos the pancreas was removed. The operation was completed at 4 15 o'clock on the afternoon of May 2. Upon catheterization at 10 00 p m of the same day 10 cc of urine were obtained which contained approximately 1 gram of dextrose. The 150 cc of urine obtained by catheterization the following morning at 10 30 yielded 5 4 grams dextrose.

EXPERIMENT 2 A bull bitch was depancreatized. At 12 30 p m, May 8, the operation had been completed. In the 100 cc of urine obtained by catheterization two hours subsequent to the operation 7 33 grams of dextrose were eliminated.

These observations indicate that dextrose may appear in significant quantities in the urine within a very few hours after removal of the pancreas, in some instances after an interval of two hours. It would seem therefore that extirpation of the pancreas has an almost *immediate* effect in evoking glycosuria, that there is little or no significant latent period, which would conform to the blood sugar content values obtained under practically comparable conditions.<sup>1</sup> These experiments also indicate that the pancreas removal was sufficiently complete to insure the speedy initiation of pancreatic diabetes.

*The prevention of pancreatic diabetes by the subcutaneous injection of hydrazine*

The observation that hydrazine will almost invariably establish a condition of hypoglycaemia in the dog at once suggested the possibility that by this method pancreatic diabetes could be pre-

<sup>1</sup> Underhill *This Journal*, 1, p 113, 1905-06



vented It is well known that during this disturbance of carbohydrate metabolism the sugar content of the blood is above the normal The assumption has been made that in this form of diabetes sugar appears in the urine only after the dextrose content of the blood has risen to a certain unknown point beyond which the kidneys become permeable to the carbohydrate Theoretically at least if the sugar content of the blood could be kept below this point no dextrose should appear in the urine In other words the appearance of sugar in the urine would depend upon which of the two factors at issue, namely, hydrazine with its hypoglycaemia producing action or the unknown force set free by removal of the pancreas exerting an influence toward blood sugar accumulation, would display the greater activity

With the combination of pancreas removal and hydrazine injection it is therefore possible to imagine at least three conditions in which theoretically glycosuria may not appear In the first place, the hydrazine effect may be very much stronger than the influence of pancreas removal, in which event sugar would not be eliminated in the urine Secondly, the two effects may exactly neutralize each other causing a normal blood sugar content and thus prevent glycosuria Finally, the effect induced by removal of the pancreas may be greater than the hydrazine influence, but the action of the latter may be sufficient to prevent accumulation of sugar in the blood to the point at which sugar elimination begins

These theoretical considerations have been put to the test The details of these experiments may be found in the following protocols

EXPERIMENT 3 Dog 9 On May 9 at 4 00 p m a full-grown terrier bitch of 8 kilos received a subcutaneous injection of 16 cc of a 2.5 per cent hydrazine sulphate solution (50 mgms per kilo) At 11 15 a m, May 11, the pancreas had been entirely removed, the operation being performed under ether anaesthesia The animal was wrapped in absorbent cotton and placed in a cage in a warm room The urine (60 cc) obtained by catheterization at 3 30 yielded no trace of reducing substance with Benedict's solution<sup>1</sup> A similar negative result was given with 40 cc urine obtained by catheterization at 5 15 p m Upon catheterization at 11 30 p m 65 cc of urine were drawn which contained no dextrose When left the dog was in good condition and responded to petting The following day at 8 00

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<sup>1</sup> Benedict *This Journal*, v, p 485, 1908

a m the animal was found dead 40 cc of urine taken from the bladder gave negative tests with Benedict's solution Autopsy revealed entire absence of pancreas, the liver presented the typical pale appearance of hydratinized dogs

EXPERIMENT 4 Dog 10 At 1:00 p m, May 13, 20 cc of a 2.5 per cent solution of hydrazine sulphate were administered hypodermically to a well nourished bitch of 10 kilos On May 15 the operation for pancreas removal was completed at 11:30 a m Immediately after the operation the animal was given a subcutaneous injection of 150 cc 0.9 per cent sodium chloride solution to supply fluid to the tissues The urine (50 cc) obtained by catheterization at 2:30 p m did not reduce Benedict's solution At 6:00 p m catheterization yielded 45 cc urine which contained no reducing substance Benedict's test was also negative with the 110 cc of urine obtained by catheterization at 11:30 p m The following morning 150 cc of urine obtained at 9:00 a m by use of the catheter contained 3.65 grams dextrose The animal was immediately given 10 cc hydrazine hypodermically At 12:30 p m the dog voided urine which gave a faint test for sugar The quantity present was too small, however, to be estimated with the polariscope Only 5 cc of urine were obtained by catheter at 3:00 p m and this gave a negative Benedict's test At 6:00 p m the few cubic centimeters of urine obtained did not reduce Benedict's solution The animal at this time appeared to be failing rapidly It was apparent that the kidneys were not functioning as well as normally, therefore, the dog was given a subcutaneous injection of 100 cc 0.9 per cent sodium chloride solution The 10 cc of urine procured at 11:15 p m failed to give any evidence of reducing compounds The following morning the dog was found dead The bladder was empty The liver was very light colored No evidence of pancreatic rests could be seen

EXPERIMENT 5 Dog 6 A well-fed bitch of 11.5 kilos received a subcutaneous injection of 23 cc of a 2.5 per cent hydrazine sulphate solution on the afternoon of April 24 The pancreas was removed April 28, the operation being completed at 4:30 p m A few hours previous to the operation 10 cc hydrazine (2.5 per cent solution) were given The urine obtained at 12:30 a m April 29 did not reduce Benedict's solution At 9:00 a m of the same day the dog was found dead No reducing substance was present in the 100 cc of urine found in the cage bottle The liver presented the typical light colored appearance No trace of pancreas was in evidence

The results of these observations warrant the conclusion that *in the quantities employed the subcutaneous administration of hydrazine is capable of preventing the appearance of sugar in the urine of depancreatized dogs* The non-appearance of sugar in the urine under the experimental conditions can not be ascribed to a latent period attendant upon pancreas removal for in the control experiments given above glycosuria promptly follows extirpation of the pancreas

*The sugar content of the blood of hydrazinized dogs after pancreas extirpation*

For the correct interpretation of the above mentioned results obtained with hydrazinized dogs after pancreas removal some knowledge of the behavior of the blood sugar is imperative. Such data would afford a much better idea of the changes which occur in sugar metabolism than can be gained by a study of the appearance of sugar in the urine alone. It is true that preliminary experiments previously reported have demonstrated the action of hydrazine in causing hypoglycaemia but it does not necessarily follow that the same activity prevails after pancreas removal. It can be conceived for instance that hydrazine may have at least a two-fold activity—one causing a temporary hypoglycaemia, the other an action rendering the kidney less permeable to sugar poured into the blood after pancreas removal, in a manner opposite to that which has been ascribed to phloridzin. Improbable though this seems, it was necessary to test the matter experimentally.

TABLE I

*The sugar content of blood of hydrazinized dogs after pancreas removal*

NUMBER OF DOG AND BODY WEIGHT	SUBCUTANEOUS INJECTION OF HYDRAZINE SULPHATE	SUGAR CONTENT OF BLOOD IM- MEDIATELY BE- FORE PANCREAS REMOVAL	SUGAR CONTENT OF BLOOD AFTER PANCREAS REMOVAL		
			After half hour	After three hours	After six hours
	<i>mgms per kilo</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
13 * 12 kilos	50	0.029	0.031	0.016	
14 * 16.4 kilos	50	0.09	0.15	0.13	0.12
15 * 8.5 kilos	50	0.04		0.03	0.01

\*No sugar appeared in the urine throughout the experiment

In the experiments reported in Table 1 the animals were subjected to the action of hydrazine for a period of two days after which the pancreas was removed. The blood sugar was determined in arterial blood drawn from a femoral artery. The observations reveal at least two indications (a) Hydrazine maintains its typi-

cal effect upon the blood sugar by keeping it far below normal in spite of the opposite accumulative tendency characteristic of pancreas extirpation, and (b) in animals in which hydrazine does not appear to greatly reduce the blood sugar, its influence is still sufficient to keep the content of blood sugar below the point at which the kidney becomes permeable to it

In presenting these data the realization is borne in upon us that the duration of these experiments is not very great. They represent, however, the extreme limit of time that our animals could withstand the combined action of hydrazine poisoning, pancreas removal and extensive hemorrhage. The latter alone is quite detrimental since for exact determination of small quantities of sugar in the blood fairly large amounts of blood should be employed. We have used between twenty and thirty grams of blood for each estimation.

*The inhibition of pancreatic diabetes in dogs by subcutaneous injections of hydrazine*

The non-appearance of sugar in the urine of depancreatized dogs previously subjected to the influence of hydrazine is possibly open to the criticism that the pancreas extirpation was not sufficiently complete to have invariably induced glycosuria. Although we consider this criticism hardly valid inasmuch as we have never failed to produce glycosuria after pancreas removal in normal dogs it is nevertheless true that the inhibition of pancreatic diabetes by hydrazine would be of far more significance than the prevention only.

Details of the experiments planned to demonstrate this feature of the investigation are to be found in the following protocols:

EXPERIMENT 6 Dog 7 The pancreas was removed from a full-grown bitch of 5 kilos on May 2. The operation was completed at 4.15 p. m. Urine collected by catheter at 10.00 p. m. contained about 1 gram of dextrose. The urine contained in cage bottle and that obtained up to 10.30 a. m., May 3, was found to contain 5.4 grams dextrose. At this time 5 cc. 2.5 per cent solution hydrazine sulphate were injected subcutaneously. At 3.30 p. m. 50 cc. of urine obtained by catheterization showed a content of 2.83 grams dextrose. A second injection of hydrazine (10 cc. of the above solution) was administered. The urine (250 cc.) yielded from this time up to 10.30 a. m., May 4, showed the presence of 1 gram dextrose. Catheterization at 3.30 p. m. furnished 45 cc. of urine which gave a very faint

test with Benedict's solution The quantity of dextrose was too small to be indicated by the polariscope The animal was catheterized at 6 00 p m and at 9 00 p m and the quantity of urine obtained each time consisted of only a few drops which gave a negative test with Benedict's solution The animal was found dead the next morning

From this experiment it is evident that hydrazine had a very decided influence upon the pancreatic diabetes, at first by greatly diminishing the quantity of dextrose eliminated and finally by completely inhibiting its appearance in the urine This result may perhaps be seen better from the following consideration The sugar eliminated from the time of operation on May 2 to 3 30 p m on May 3 amounted to 9.23 grams, that excreted from May 3 at 3 30 p m to May 4 at 3 30 p m was only 1 gram After 3 30 p m on May 4 the urine was sugar-free The decrease from 9.34 grams of dextrose in approximately twenty-four hours to 1 gram during the succeeding day shows the remarkable rapidity with which hydrazine must accomplish its action From the rapid falling off in urine secretion it is also evident that hydrazine must exert a distinct influence upon the kidney secretion

EXPERIMENT 7 Dog 11 On May 16 the pancreas was removed from a bitch of 7 kilos the operation being completed at 11 00 a m As soon as the animal was under the influence of ether preparatory to the operation a subcutaneous injection of 50 mgms hydrazine sulphate per kilo was given Catheterization at 12 30 p m yielded 40 cc of urine which was sugar-free The 30 cc of urine obtained at 3 30 p m yielded a small quantity of dextrose At 6 00 p m the dog was again catheterized and the 20 cc of urine yielded no reducing body At this time 100 cc 0.9 per cent sodium chloride were injected subcutaneously From the catheterization at 11.20 p m 30 cc of urine obtained gave no evidence of the presence of dextrose On the following day 100 cc of urine were obtained at 9 00 a m which showed the presence of the merest trace of dextrose At this time 10 cc of hydrazine sulphate (2.5 per cent solution) were given The urine furnished at 3 30 p m and 5 30 p m gave evidence of a mere trace of reducing substance At 11 45 p m the urine (10 cc) obtained by catheterization furnished evidence of the presence of dextrose in the slightest degree only, by Benedict's test the merest precipitate formed on cooling The animal was still living at 10 00 a m, May 18, but was in a somewhat comatose condition About 5 cc of urine obtained from the bladder contained no reducing substance The animal died during the morning

The results of this experiment demonstrate that the influence of hydrazine was almost sufficient to completely inhibit the elim-

ination of sugar in the urine. At one time the inhibition was complete, but during the night of May 16 it is evident that the action of hydrazine was insufficient to overcome the sugar accumulative power of the depancreatized dog and sugar was found in the urine, its appearance finally being prevented by a second dose of hydrazine.

EXPERIMENT 8 Dog 25 From a bitch of 12 kilos the pancreas was removed resulting in the elimination of 11.20 grams of dextrose for the succeeding twenty-four hours. Then a subcutaneous injection of 20 cc hydrazine sulphate in a 2.5 per cent solution was administered. Two hours later the urine excreted held 0.69 gram dextrose. An hour later 11 cc of urine obtained by catheterization yielded the merest trace of dextrose, too small to be indicated by the polariscope. Six hours after the hydrazine administration the urine was sugar-free.

The data submitted justify the conclusion that *hydrazine administered to dogs during pancreatic diabetes is capable of completely inhibiting the elimination of sugar by the kidney*.

*How much hydrazine is necessary to prevent pancreatic diabetes and how long does its action persist?*

The importance of the determination of the quantity of hydrazine necessary to prevent pancreatic diabetes is obvious. How long the peculiar action of the drug endures is a question also particularly worthy of investigation. These topics have as yet been considered in the crudest manner only and hence the results thus far obtained do not allow us to make more than the most general statements.

To find out the limit of time during which a single subcutaneous injection of 50 mgms hydrazine sulphate per kilo is still capable of exerting its preventive action toward pancreatic diabetes, the following experiment was carried through.

EXPERIMENT 9 On June 24 a 12 kilo bitch received the usual injection of hydrazine. Four days later the pancreas was removed. Within a few hours after the operation the appearance of sugar in the urine in fairly large quantities (10 to 12 grams per day) was noted. The animal lived two days after the operation.

From this result it is apparent that a single subcutaneous injection of 50 mgms hydrazine sulphate per kilo body weight is not

capable of preventing the appearance of sugar in the urine of dogs deprived of the pancreas four days after the hydrazine administration. The time during which this influence prevails must lie, therefore, somewhere between two and four days.

A single experiment to test the effect of smaller doses of hydrazine is detailed in

EXPERIMENT 10 Dog 26 On July 8 a bitch of 17.4 kilos received a subcutaneous injection of 25 mgms hydrazine sulphate per kilo. Two days later the pancreas was removed. In this experiment it was necessary to employ a small quantity of morphine in order to anesthetize the animal. The pancreas had been removed at 11.30 a. m. and 4.3 grams dextrose were found in the urine at 2.30 p. m. At 5.30 p. m. the urine held 3.2 grams dextrose. A second injection of the same dose of hydrazine was now given. The dog was found dead at 9.00 a. m. the following morning. From the bladder 30 cc. of urine were obtained which reduced Benedict's solution.

This observation indicated that a single injection of 25 mgms hydrazine sulphate per kilo is insufficient to prevent the elimination of dextrose in the urine of dogs deprived of the pancreas two days after the initial dose of hydrazine.

*A tentative hypothesis to account for the action of hydrazine upon sugar metabolism*

Porges<sup>1</sup> has demonstrated that adrenal extirpation as well as adrenal insufficiency exemplified in Addison's disease leads to a significant hypoglycaemia and a disappearance of glycogen from the liver. Phosphorus<sup>2</sup> produces the same effects and Neubauer and Porges<sup>3</sup> have ascribed to phosphorus an influence upon the adrenals leading to an insufficiency of adrenal secretion, presumably adienalin, thereby causing the liver glycogen to disappear and the blood sugar content to decrease. This theory was based upon experiments in which Neubauer and Porges failed to obtain evidence of adienalin in extracts of the adrenals after phosphorus administration.

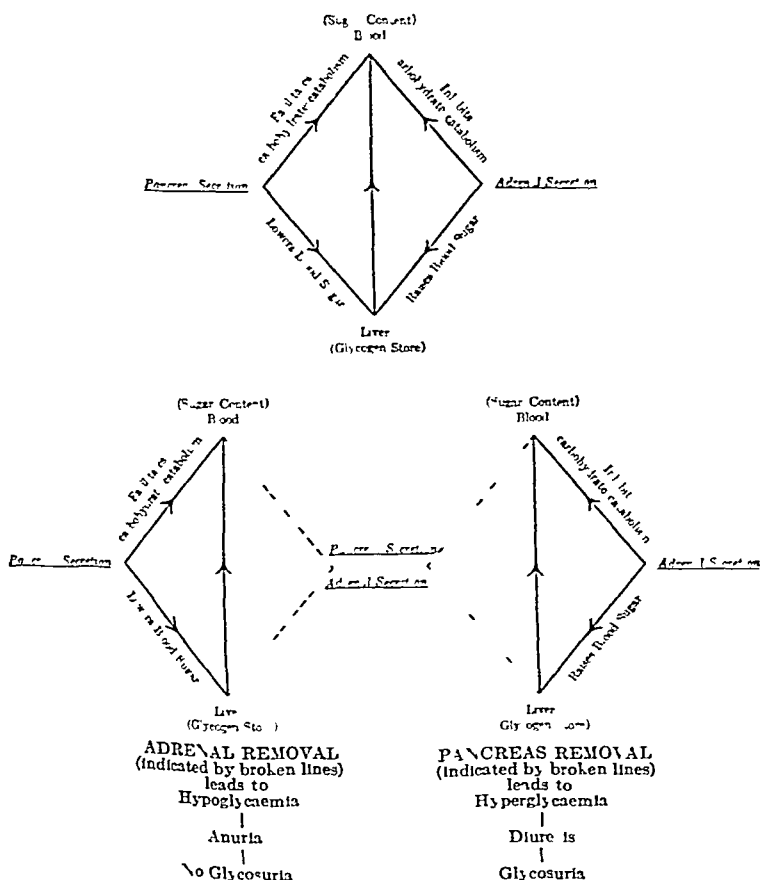
<sup>1</sup>Porges *Zeitschrift für Klinische Medizin*, lxx, p. 341, 1910, lxx, p. 143

<sup>2</sup>Frank and Isaac *Archiv für experimentelle Pathologie und Pharmacologie*, lxxv, p. 274, 1911

<sup>3</sup>Neubauer and Porges *Biochemische Zeitschrift*, xlviii, p. 290, 1911

Since phosphorus and hydrazone are now observed to be alike in their effect upon blood sugar content and liver glycogen disappearance, it is reasonable to assume that the influence of the two might possibly be directed upon the same object, namely, upon the adrenal. The explanation as to the manner in which adrenal secretion influences carbohydrate metabolism is lacking. As a working hypothesis we have made use of the following scheme of interaction

DIAGRAM INDICATING INFLUENCE OF THE ORGANS ON THE METABOLISM OF CARBOHYDRATE





In the first place we have assumed that there is an interrelation between the pancreas, the adrenals and the liver by the equilibrium of which the sugar content of the blood is kept practically constant. To accomplish this the pancreas is assumed to pour an internal secretion into the blood which tends to increase carbohydrate catabolism, hence to diminish the blood sugar. This influence we have called the pancreas secretion factor. On the other hand the adrenal is assumed to give an internal secretion to the blood which has the opposite effect, namely, it has a tendency to check carbohydrate catabolism, hence leads to an accumulation of sugar in the blood. This action we have designated the adrenal secretion factor. According to this idea of what occurs to keep blood sugar content normal, the quantity of glycogen which will be taken from the liver's store to accomplish this purpose is entirely dependent upon which of the two factors is predominant at any given moment.

If we apply these ideas to pancreas extirpation or adrenal removal, the effects which should theoretically follow according to our scheme agree perfectly with recorded observations which for the most part have universal acceptance.

When the pancreas has been removed from an animal our hypothesis assumes that the pancreas factor is taken away, in other words what we have called the influence that "facilitates carbohydrate catabolism" is lost. Consequently the blood sugar equilibrium is upset because the adrenal factor, which "inhibits carbohydrate catabolism" is no longer counterbalanced by the pancreas factor, the influence normally furnished by the pancreas. Without the check of the pancreas upon it the adrenal factor now has full play, there is less carbohydrate catabolism than normally, sugar, poured out of the liver's store of carbohydrate, accumulates in the blood (hyperglycaemia), diuresis is induced in the effort to eliminate the sugar through the kidneys, and then glycosuria is in evidence. If this condition of affairs is maintained sufficiently long the liver will be more or less depleted of its glycogen owing to the body's vain effort to furnish sufficient energy for its needs.

On the other hand, removal of the adrenals from a normal animal results in a series of events of the opposite order. The power which has a tendency to "inhibit carbohydrate catabolism," the adrenal factor, has been lost, hence the pancreas factor holds

full sway, resulting in the rapid catabolism of all available carbohydrate material even to the depletion of the liver glycogen, leading to hypoglycaemia, fall of blood pressure, hence anuria, and no glycosuria

Extirpation of both pancreas and adrenals should yield results in correspondence with which of the organs was first removed. If the adrenals were extirpated first hypoglycaemia should be in evidence, if the pancreas were removed before the adrenal, hyperglycaemia should prevail. The extirpation of both would ultimately mean a complete upset of the sugar regulation of the organism.

If we assume that hydrazine acts in a manner similar to the action ascribed to phosphorus it is evident that here hydrazine activity upon the blood sugar content would be equivalent to partial or complete extirpation of the adrenals. Hence according to our tentative hypothesis hydrazine may prevent pancreatic diabetes by its inhibition or at least suppression of adrenal function. Viewed from another standpoint it is possible that hydrazine has an action upon sugar metabolism entirely similar to that exerted by the internal secretion of the pancreas. According to this idea injections of hydrazine cause hypoglycaemia by increasing the efficiency of the pancreatic secretion or by augmenting its output. All the results thus far obtained could be interpreted upon this basis.

Assuming that hydrazine does exert some inhibitory influence upon adrenal secretion one might expect perhaps to obtain some indication of this by testing adrenal extracts furnished by hydrazinized dogs for the generally accepted active principle of adrenal secretion, namely, adrenalin.

*Is the secretion of adrenalin inhibited by hydrazine administration?*

The communication of Neubauer and Porges<sup>1</sup> demonstrating the absence of adrenalin in the adrenals as a sequel to phosphorus administration would lead one to infer that possibly hydrazine has a similar influence, more especially as both these agents have the same action upon the blood sugar causing it to be significantly decreased. The exhibition<sup>2</sup> of adrenalin in the adrenals is usu-

<sup>1</sup>Neubauer and Porges *loc cit*

<sup>2</sup>cf Biedl *Innere Secretionen*, for literature to color reactions

ally made by color reactions, among which may be mentioned the development of a red color on the addition of mercuric chloride, or green coloration caused by adding a neutral solution of ferric chloride to aqueous extracts of these organs

In our first attempts to gain evidence of the presence of adrenalin in watery extracts of the adrenals by the above mentioned color reactions we were unsuccessful. It soon developed, however, that the accompanying turbidity, presumably of a protein nature, was the cause for our failures, for if the filtrates could be obtained water clear the reactions proved to be very delicate. Our final method for demonstrating the presence of adrenalin consisted in grinding the adrenals to a pulp in a mortar with fine sand and a very little water or physiological salt solution. This mixture was filtered and the turbid filtrate clarified by the addition of just enough mercuric chloride to precipitate the protein (usually two or three drops of a 10 per cent solution). The clear filtrate, on the addition of a little more mercuric chloride, soon yielded the pink or red color characteristic of adrenalin. The development of the coloration was much more rapid and intense if the solution was warmed somewhat. The green color reaction with ferric chloride was obtained in a similar manner, the preliminary addition of mercuric chloride for the purpose of clarifying the turbid filtrate being without any apparent detriment to the reaction. In fact, so little mercuric chloride was added that it is probable that practically all of it was removed in combination with the precipitate.

With this method the extracts of the adrenals taken from seven normal dogs showed the presence of significant quantities of adrenalin.

*Dogs poisoned with hydrazine, the dose of which was in some instances large enough to kill the animals within twenty-four hours, yielded adrenals giving apparently just as strong adrenalin reactions as normal animals.* This result makes it evident, therefore, that even if hydrazine does have an inhibitory influence upon adrenal production the inhibition is not complete, it is a quantitative influence.

## CONCLUSIONS

Removal of the pancreas from normal dogs may be followed by the appearance of sugar in the urine within a period of two hours

Glycosuria fails to manifest itself after pancreas extirpation in dogs that have received previous injections of hydrazine. In general this effect is produced by a single subcutaneous injection of 50 mgms per kilo hydrazine sulphate. The influence of the hydrazine administration lasts between two and four days. A single injection of 25 mgms per kilo hydrazine sulphate does not prevent sugar elimination in the urine, under the experimental conditions, after pancreas removal.

The blood sugar content of animals treated with hydrazine and then depancreatized remains below the normal, or at least hyperglycaemia is not in evidence.

Hydrazine introduced into dogs during pancreatic diabetes is capable of completely inhibiting the excretion of sugar by the kidney.

After hydrazine administration the presence of adrenalin in the adrenals may still be demonstrated. In this respect hydrazine differs from phosphorus.

A tentative hypothesis to account for the phenomena is presented.

We are greatly indebted to Professor Lafayette B Mendel for aid in some of the operations for pancreas extirpation and for criticism of the manuscript.



# AN IMPROVED APPARATUS FOR THE DETERMINATION OF AMINO GROUPS

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(Received for publication, August 15, 1911)

Recently Van Slyke<sup>1</sup> has proposed a method for the estimation of alphatic amino groups, at the same time describing an apparatus for carrying out the determinations. The apparatus herein described is the result of our experience with the original apparatus of Van Slyke.

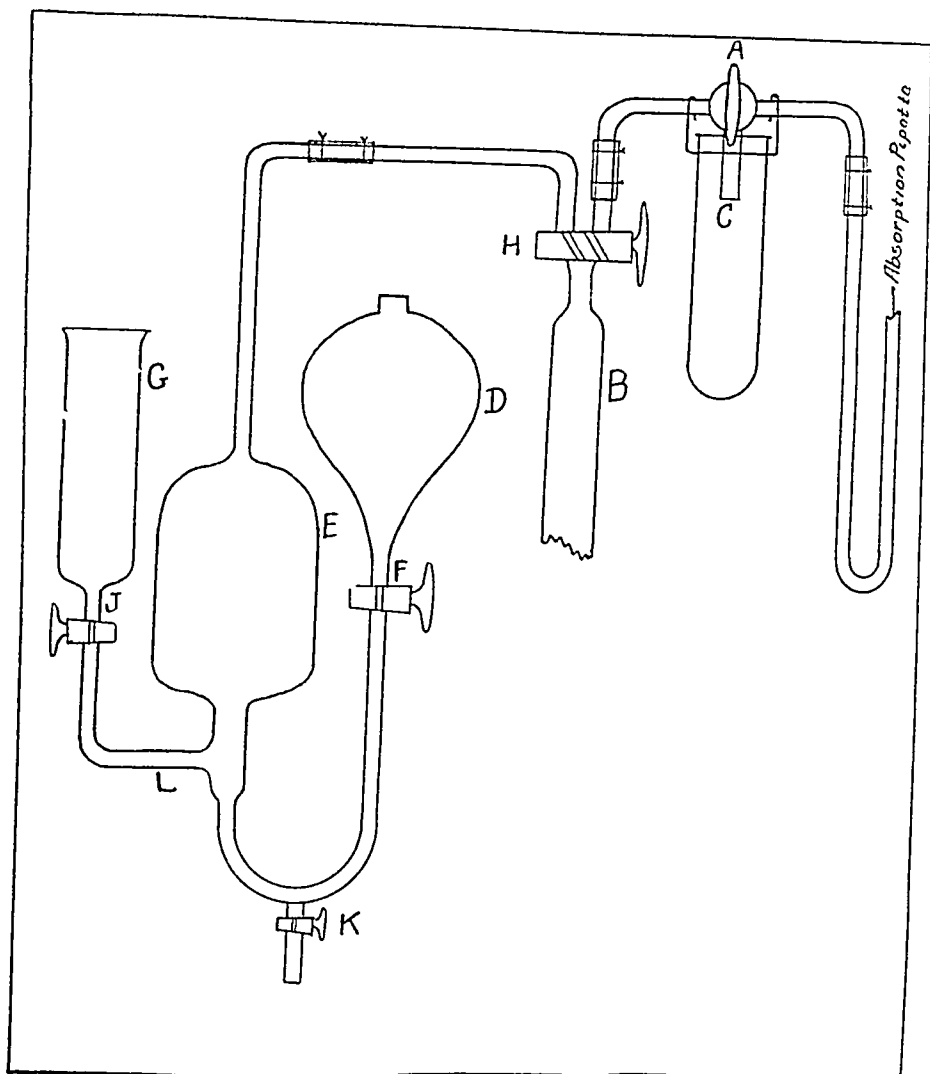
The advantages of the new method over the old may be enumerated as follows: (1) Simpler design (2) Less likelihood of leakage (3) Greater permanence of installation. Once the apparatus is set up it can be used indefinitely without disconnecting it at any point, either during the experiment or after its completion.

The constructive details of the apparatus are quite evident from the figure. All tubing is a medium bore capillary tube, a fine capillary is of no special value. The manipulation is as follows:

Draw the liquid from the absorption pipette so that it comes through the stop-cock *A* of the capillary three-way stop-cock. Then turn this stop-cock so as to make connection between the burette *B* and the air at *C*. Pour 28 cc. of the potassium nitrite solution in the reservoir *D*, from which it is drawn into *E* by lowering the levelling bulb to burette *B*. It is advisable to leave a little of the liquid above the cock *F*. Discharge the air in the burette *B* through *A*. It is not necessary to fill the capillary tube with liquid. Pour the solution to be analyzed into *G*. Then pour 7 cc. of glacial acetic acid into *D*, from which it is allowed to enter *E* by lowering the levelling bulb of the burette. Do not let

<sup>1</sup>This *Journal*, ix, p. 155, 1911.

all of the acid run below *F*. The evolving gas soon displaces all of the air in *E*. When about 45 to 50 cc of gas have been collected in the burette, close *H* and open *F*. A sufficiently large



gas space is soon formed in *E*. Meanwhile drive all of the air out of the burette, this time filling the capillary so that a few drops of the liquid run out of *A* into the test tube over *C*. Close *H* and turn *A*, so as to connect the absorption pipette with the burette. Close *F* and draw the liquid from *G* to *E*, being careful not

to run it below *J*. Close *J*, wash *G* with a little water which is then run into *E*. Repeat two or three times. After the action has proceeded five minutes, draw liquid from *D* into *E* until it rises to *H*. Then force the gas from the burette into the absorption pipette, so that the acidulated water entirely fills the capillary of the pipette. If the pipette is shaken while the gas is passed into it, the time of absorption can be greatly reduced. The unabsorbed gas is run back into the burette, this time drawing the permanganate to tap *H*. The rest of the method is the same as in Van Slyke's paper.

To clean the apparatus force the liquid through *K*. Distilled water is admitted through *D* and *G* into *E*, which can be cleaned with two or three washings. The reaction vessel is conveniently supported upon a grooved cork at *L*, from which it is easily removed to shake the apparatus from time to time, and as easily replaced. An additional tube for amyl alcohol is unnecessary. The amyl alcohol could be admitted before the potassium nitrite solution through *D*, or later through *G*.





# A NOTE ON SHAFFER'S METHOD FOR THE DETERMINATION OF $\beta$ -OXYBUTYRIC ACID

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(Received for publication, August 25, 1911)

A few years ago Shaffer proposed a method for the quantitative estimation of  $\beta$ -oxybutyric acid in urine,<sup>1</sup> which depends upon the conversion of the acid to acetone by distillation with potassium dichromate in an acid medium, after preliminary removal of interfering substances by an excess of basic lead acetate and ammonia

Embden and Schmitz,<sup>2</sup> in a discussion of this method, state that satisfactory results were obtained on normal urines to which  $\beta$ -oxybutyric acid was added, but that with urines containing both sugar and  $\beta$ -oxybutyric acid duplicate determinations varied widely. They report also, contrary to Shaffer, that glucose itself, when distilled under the conditions prescribed, yields iodine-binding compounds to such an extent that the method is inapplicable to sugar-containing urines. Reports from several other laboratories have shown dissatisfaction because of a discrepancy between duplicate determinations.

These criticisms have prompted us to report our results, which have proved entirely satisfactory when the method is followed with the modifications suggested below.

Contrary to the findings of Embden and Schmitz and in agreement with Shaffer, our results show that in urines containing considerable sugar, the amounts of iodine-binding compounds formed are extremely small and are not sufficient to vitiate results. Nor-

<sup>1</sup>This Journal, v, p 211

<sup>2</sup>Abderhalden's *Handbuch d. biochem. Arbeitsmethoden*, III, part 2, p 936

mal urines, as is well known, give iodine-binding compounds rarely exceeding an amount corresponding to 0.1 gram of  $\beta$ -oxybutyric acid for a twenty-four-hour specimen. The following are samples from determinations on normal urines and on the same urines in which 2 per cent of sugar was present during distillation. The results are given for duplicate analyses and calculated for 2000 cc of urine.

	Gram
1 a Normal urine	{ 0.107
	{ 0.107
b Same urine + 2 per cent of glucose	{ 0.134
	{ 0.134
<i>Increase due to glucose</i>	0.027
2 a Normal urine	{ 0.072
	{ 0.072
b Same urine + 2 per cent of glucose	{ 0.098
	{ 0.107
<i>Increase due to glucose</i>	0.035

The difficulty, therefore, with sugar-containing urines lies not in the fact that iodine-binding compounds are formed, but rather that glucose, if present, is much more readily oxidized than  $\beta$ -oxybutyric acid by the dichromate, and as a result the oxidizing agent is rapidly used up and may not be present in the concentration required for the most rapid and complete conversions of the acid present, the determinations of which are consequently low and irregular.

As a matter of fact, and this is a point that Embden and Schmitz have apparently overlooked, the interference by glucose can be entirely disregarded, for the preliminary treatment of urines by ammonia and basic lead acetate in excess almost quantitatively removes this sugar, as well as glycuronates. We have examined the filtrates of a number of urines so treated, some originally containing as high as 10 per cent of sugar, and have never found higher than 0.1 per cent of glucose present in the filtrates.

We wish, therefore, to emphasize the importance of the addition of basic lead acetate in excess. As this is only assured by testing the filtrate, we have embodied as one modification the addition of sulfuric acid, which shows an excess of lead in solution, and at the same time removes it by precipitation.

Shaffer advised the addition of 400 to 500 cc of 0.1 per cent to 0.5 per cent solution of dichromate through a dropping funnel. We have found that 5 cc of 5 per cent dichromate, amounting to 0.25 gram of the substance, may be added at once at the beginning of the distillation and smaller amounts (1 to 5 cc) later, depending upon the appearance of a greenish color. The contents of the flask should always be kept a decided yellow. Rarely more than 10 cc of dichromate is needed.

The following procedure has given satisfactory results in a large number of determinations on urines containing varying amounts of sugar and  $\beta$ -oxybutyric acid. It is essentially the method of Shaffer, modified to insure the addition and subsequent removal<sup>1</sup> of an excess of basic lead acetate and also to simplify the procedure by the addition of more concentrated dichromate solution.

From 25 to 250 cc of urine<sup>2</sup> are measured into a 500 cc glass-stoppered graduate. An excess<sup>3</sup> of basic lead acetate and 10 to 20 cc of concentrated ammonia are added. After diluting to 500 cc the mixture is thoroughly shaken and filtered. Three-hundred cubic centimeters of the filtrate are put in a 500 cc glass-stoppered graduate, neutralized with concentrated sulfuric acid, and again made up to 500 cc and filtered. An aliquot part of this filtrate, usually about 200 cc, is placed in an 800 cc Kjeldahl flask.<sup>4</sup> Fifteen cubic centimeters of concentrated sulfuric acid and a little talcum are added, and water to 500 cc. The mixture is then distilled,<sup>5</sup> about 250 cc of distillate being collected.

<sup>1</sup> Removal of excess of lead acetate is necessary to prevent bumping during distillation. If there is only a slight precipitate of lead sulphate it may be allowed to remain.

<sup>2</sup> When the urine gives a strong reaction for diacetic acid with ferric chloride, and from 5 to 10 gms of  $\beta$ -oxybutyric acid are expected, from 25 to 50 cc of the urine will be sufficient. Where little  $\beta$ -oxybutyric acid is expected, from 150 to 250 cc should be used.

<sup>3</sup> Basic lead acetate may be added either dry or as a saturated solution. Three volumes of the latter to one of urine will furnish the needed excess with urines containing as high as 5 per cent of glucose. It is always well to test a small sample of filtrate with sulfuric acid, however, in order to be sure of the required excess.

<sup>4</sup> The flask is conveniently marked to show volumes of 600, 400 and 300 cc and should be fitted with a dropping tube.

<sup>5</sup> The condenser should be well cooled by a rapid stream of cold water and a little cold water should be placed in the collection flask.

This distillate (A) contains the volatile acids and acetone, prepared and from diacetic acid. The former are removed by distilling again after adding 10 cc of 10 per cent sodium hydroxide and a little talcum. The resulting distillate is titrated with standard iodine and sodium thiosulfate.<sup>1</sup>

The residue of urine and sulfuric acid is diluted to 600 cc and 5 cc of a 5 per cent solution of potassium dichromate is at once added. The mixture is then distilled down to 300 cc. Water is added through the dropping tube to 500 cc and distillation continued down to 300 cc again. In this way 500 cc of distillate is collected (Distillate B). Additional 5 per cent bichromate is run in, a few cubic centimeters at a time whenever the solution commences to turn green, showing a reduction of considerable amount of the dichromate. The volume should never be allowed to decrease below 250 cc.

Distillate B is redistilled after adding 20 cc of 3 per cent hydrogen peroxide and 10 cc of 10 per cent sodium hydroxide. About 300 cc are collected and this is titrated with the standard iodine and thiosulfate as usual. This gives the amount of  $\beta$ -oxybutyric acid present in terms of acetone.

The following are samples of the duplicate results obtained by this method on sugar-containing urines. The conversion of  $\beta$ -oxybutyric acid was complete in 500 cc, continued distillation showing no iodine-binding compounds.

		ACETONE AND DIACETIC ACID	$\beta$ OXYBUTYRIC ACID
		grams	grams
1	Twenty-four-hour urine }	3.870	13.05
		3.784	13.12
		2.21	8.36
2	Twenty-four-hour urine }	2.34	8.49
		2.92	13.20
3	Twenty-four-hour urine }	2.85	13.13

<sup>1</sup> If 103.4  $\frac{N}{10}$  iodine and thiosulfate are used, 1 cc equals 1 mg of acetone or 1.794 mg of  $\beta$ -oxybutyric acid.





# THE CATALASE OF SEA URCHIN EGGS BEFORE AND AFTER FERTILIZATION WITH ESPECIAL REFERENCE TO THE RELATION OF CATALASE TO OXIDATION IN GENERAL

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(Received for publication, September 2, 1911)

The function of the enzyme catalase remains obscure despite repeated efforts to solve the question. The literature upon this subject has been reviewed recently in the monograph of Kastle<sup>1</sup> and new contributions are constantly appearing. It is not our purpose to enter upon this discussion but simply to test one side of the problem, namely, whether any relationship exists between oxidative processes in general and the power of the tissues to decompose hydrogen peroxide.

The sea urchin's egg offers an advantageous means of approaching this phase of the problem, since it has been shown by Warburg<sup>2</sup> and J. Loeb<sup>3</sup>, working with two different genera of sea urchins, that these eggs after fertilization consume several hundred per cent more oxygen than before.

A series of experiments very similar to those herewith reported was conducted by E. P. Lyon.<sup>4</sup> This author determined the catalytic activity of Echinoderm eggs before and after fertilization, etc. The following paragraphs are quoted from his report.<sup>5</sup>

"Starting with equal suspensions of fertilized and unfertilized eggs, it was observed that, soon after the addition of distilled water, the unfertilized

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<sup>1</sup>Hygienic Laboratory, Bulletin No. 59, 1909

<sup>2</sup>*Munch med Wochenschr*, lviii, p. 298, 1911

<sup>3</sup>*Arch f Entwicklungsmechanik d Organismen*, xxi, p. 658, 1911

<sup>4</sup>*Amer Journ of Physiol*, xxv, p. 199, 1909

<sup>5</sup>Lyon *Loc cit*, p. 207



eggs in some experiments might split peroxide as rapidly as the fertilized. Indeed sometimes the action of the unfertilized eggs after treatment with distilled water exceeded for a time that of fertilized. The differences were not striking, however, and some little time after the addition of distilled water, in the clearest experiments the fertilized again exceeded the unfertilized eggs in catalytic power. But the differences here were not nearly so great as those between the normal fertilized and unfertilized eggs in sea water. In other words, the effect of the addition of distilled water was a real or apparent increase of catalase in both fertilized and unfertilized eggs, but in the latter much more than in the former."

He concludes "that if the entire egg of *Tozopneustes* or *Arbacia* be treated with hydrogen peroxide, much more oxygen is set free by eggs which have been fertilized a few minutes than by unfertilized eggs. The change in the catalytic power begins about three minutes after sperm is added and reaches a maximum in about twenty minutes. No further striking change in catalase content or action is demonstrable either with each succeeding cleavage or at later stages of development."

The element of uncertainty of the first paragraph renders the author's summary less conclusive to the reader. Suffice it to say, our experiments do not confirm Lyon's findings.

In all of our experiments only fresh eggs and fresh spermatozoa were used.

#### METHOD

The usual method of determining the catalytic activity was employed. Ten cubic centimeters of a suspension of egg or sperm, etc., were placed in a salt mouth bottle in which there was a vial containing 5 cc of freshly neutralized hydrogen peroxide (Oakland, 3 per cent) and the bottle connected with a gas burette. The small vial was then overturned and the bottle containing it agitated for a period of one minute. The hydrogen peroxide at once began to decompose and the amount of gas liberated was read every fifteen seconds.

The sperm was collected in sea water. Its catalytic activity in the amounts used was found to be very low. For instance, three drops of an emulsion of sperm in sea water were added to 10 cc of distilled water and kept as a stock. Two cubic centimeters of this stock diluted with 8 cc of distilled water gave the following readings:

SECONDS	OXYGEN	OXYGEN CHECK
	cc	cc
15	0 2	0 1
30	0 2	0 3
45	0 2	0 3
60	0 3	0 4

This determination of sperm activity was corroborated repeatedly. In the above concentration its power of decomposing hydrogen peroxide is so small that it will not be considered in the experiments to follow.

Eggs were collected in sea water, strained through cheese cloth and washed three or four times with large amounts of fresh sea water. The washing was always performed in the refrigerator. A fixed quantity of eggs thus obtained was suspended in a definite quantity of sea water and kept as a stock. One cubic centimeter of this suspension was then diluted with 9 cc of sea water and its catalytic activity determined. The above dilution was arbitrarily chosen, since, at that strength the amount of oxygen liberated could be more readily and accurately measured. The same pipette to measure the eggs was used throughout the experiments. One was selected which permitted of a rapid discharge thus minimizing this error as much as possible. In the actual experiments, 10 cc of the stock suspension of ova were measured into each of four centrifuge tubes<sup>1</sup>. Two of these tubes contained simply the ova suspension and two were fertilized. The fertilization was always controlled microscopically. Only one to three drops of the emulsion of sperm was necessary for this purpose, and this could not effect the catalytic activity.

After centrifugation the supernatant fluid was carefully removed, first with pipette and then with filter paper, and 10 cc of distilled water added. The tubes were then agitated until the ova were proven to be disintegrated by microscopic examination. In this process it was noted that the fertilized eggs disintegrated more easily than the unfertilized ones. Two cubic centimeters of this emulsion were further diluted with 8 cc of distilled water and its catalytic

<sup>1</sup>The speed of centrifugation has no effect upon fertilization.

activity tested As a rule not only were duplicate readings made, but also duplicate experiments were carried through The experiments with unfertilized eggs will be designated A and A', those with fertilized eggs B and B' All results are expressed as cubic centimeters of oxygen

EXPERIMENT 1 Fertilization 12 minutes, microscopically, pronounced fertilization membrane, very few unfertilized eggs

SECONDS	UNFERTILIZED				FERTILIZED			
	A	A	A'	A'	B	B	B'	B
15	5 4	5 0	5 2	5 4	5 6	6 4	5 8	6 0
30	11 0	10 4	10 6	10 4	11 0	11 6	11 0	11 6
45	16 0	15 6	15 4	15 0	15 6	16 2	15 8	16 2
60	20 4	19 8	19 8	19 0	20 2	20 8	20 6	20 6

EXPERIMENT 2A Fertilization 10 minutes Fertilization membrane well developed

SECONDS	UNFERTILIZED				FERTILIZED			
	A	A	A'	A'	B	B	B'	B'
15	7 0	6 8	7 0	6 8	7 4	7 0	7 6	7 6
30	13 6	13 6	13 2	13 4	14 0	14 6	14 0	14 4
45	19 6	19 4	19 2	19 6	20 6	20 8	20 4	20 6
60	25 2	25 0	24 6	24 8	26 2	26 4	26 2	26 2

EXPERIMENT 2B Fertilization from 30 to 35 minutes, otherwise treated exactly as A Microscopically, mostly fertilization membrane, some with beginning division, others progressed as far as the 4-cell stage

SECONDS	UNFERTILIZED				FERTILIZED			
	A	A	A'	A'	B	B	B'	B'
15	7 0	6 8	7 0	6 6	6 8	6 8	6 4	6 6
30	13 6	13 2	13 4	13 6	13 0	13 0	13 4	13 0
45	19 6	19 4	19 4	19 6	19 2	18 2	19 4	19 4
60	24 6	24 6	25 0	25 0	24 6	24 2	25 2	25 0

EXPERIMENT 3A. Fertilization 10 minutes Microscopically, mostly membrane stage, few cells beginning division

SECONDS	UNFERTILIZED				FERTILIZED			
	A	A	A'	A	B	B	B	B
15	6 4	5 8	6 4	6 2	6 0	6 2	6 2	6 2
30	12 4	11 4	12 4	11 2	12 2	12 6	12 2	12 2
45	17 6	17 0	18 0	17 2	17 8	18 2	18 0	17 8
60	23 0	22 4	23 0	21 8	23 0	23 4	23 0	21 8

B Fertilization one-half hour longer than in A, i.e., 40 minutes Microscopically, the majority show fertilization membranes but many show 2- and 4-cell stage

SECONDS	UNFERTILIZED				FERTILIZED			
	A	A	A	A	B	B	B	B
15	6 0	6 4	6 4	6 4	6 4	6 2	6 2	6 0
30	12 6	12 4	12 6	12 8	12 2	11 8	12 2	11 8
45	18 6	18 6	18 4	18 4	17 6	17 4	17 2	17 4
60	24 0	23 6	23 4	23 6	23 4	22 2	22 2	22 0

EXPERIMENT 4A Fertilization 10 minutes Microscopically, membrane stage

SECONDS	UNFERTILIZED		FERTILIZED	
	A	A	B	B
15	7 8	6 8	7	7 2
30	14 0	13 6	13 4	13 6
45	20 0	19 4	19 4	19 6
60	25 4	24 8	25	25 0

## Relation of Catalase to Oxidation

B Fertilization 55 minutes Microscopically, beginning division in cells, few cells divided into 2-cell stage

SECONDS	UNFERTILIZED		FERTILIZED	
	A	A	B	B
15	7 8	7 4	7 0	7 0
30	14 8	14 0	13 8	13 4
45	20 8	20 0	19 8	19 4
60	25 4	25	25 0	24 4

C Fertilization 95 minutes Microscopically, division into 2-cell stage, few more advanced

SECONDS	UNFERTILIZED		FERTILIZED	
	A	A	B	B
15	7 8	7 6	7 2	7 2
30	15 2	14 8	13 4	13 2
45	20 6	21 0	19 2	19 0
60	26 2	26 4	24 4	24 2

In many instances the catalytic activity of the supernatant sea water was determined also 5 cc were diluted with 5 cc of distilled water The following example is quoted from experiment 2 where the supernatant fluid was tested

SECONDS	UNFERTILIZED		FERTILIZED	
	A	A'	B	B
15	0 6	0 5	0 5	0 5
30	0 8	0 8	0 8	0 9
45	1 0	0 9	1 2	1 1
60	1 1	1 2	1 4	1 5

Unfortunately the catalytic activity of the sea water itself was not determined but the results obtained with the supernatant fluid, of which the above is an example, were so uniform that the fertilization could not have exercised any influence upon its activity

Lyon noted in his experiments that the rate of evolution of the oxygen did not follow the reaction of the first order From our tables it will be seen that the rate of evolution in the time units seems to be very uniform—that is, approximately the same amount of oxygen is liberated in each 15 seconds

The above experiments show firstly, that the fertilization of the eggs does not exercise any influence whatsoever upon its catalytic activity, and secondly, since the sea urchin's egg after fertilization uses several hundred per cent more oxygen than before, it does not seem probable that the catalytic activity has any direct relation to oxidative processes in general

The explanation for the increase of catalytic activity of sea urchins' eggs treated with distilled water lies in the fact that the water liberates the catalase contained in the egg In support of this view two other observations may be cited Firstly it is known that the *Fundulus* egg is resistant to distilled water<sup>1</sup> Unfortunately the season for *Fundulus* at the Marine Biological Laboratory, Wood's Hole, Mass, where these experiments were conducted, was nearly over and only 158 ripe eggs of *Fundulus heterochtus* were obtainable These were divided into two equal parts, washed several times with sea water, and finally the sea water was carefully removed One portion was taken up in 12 cc of distilled water The other portion was diluted with 10 cc of distilled water The eggs were thoroughly mashed with a glass rod and the suspension well shaken This was then washed into the container with 2 cc more of distilled water and tested

SECONDS	A UNBROKEN EGGS	B BROKEN EGGS
15	0 1	0 7
30	0 3	0 9
45	0 3	1 2
60	0 4	1 5

The material to test fertilized eggs could not be obtained Secondly A fixed amount of human blood was removed in a white blood pipette In experiment A this was placed in 10 cc

<sup>1</sup>Loeb Arch f Entwicklungsmechanik d Organismen, xxxi, p 654, 1911

of distilled water and tested. In experiment B it was placed in 5 cc of distilled water and 5 cc of  $\frac{5}{8}$  NaCl solution were added. This laked the corpuscles. In experiment C it was placed in  $\frac{5N}{2}$  NaCl solution which did not lake the corpuscles. These were then tested with the following results

SECONDS	A	B	C
15	19 6	19 5	2 8
30	27 0	25 5	6 4
45	37 6	31 4	9 6
60	39 0	36 8	12 6

Similar results may be obtained with the sea urchins' egg as is seen in the following experiment and as Lyon has shown previously

A 0.5 cc of certain emulsion of sea urchin eggs plus 9.5 cc of sea water

B 0.5 cc of certain emulsion of sea urchin eggs plus 9.5 cc of distilled water

C 1.0 cc of certain emulsion of sea urchin eggs plus 9.0 cc of sea water

D 1.0 cc of certain emulsion of sea urchin eggs plus 9.0 cc of distilled water

SECONDS	A	B	C	D
15	1 2	3 2	2 0	6 2
30	2 4	6 0	2 4	10 6
45	3 8	9 0	4 2	15 0
60	5 2	12 0	8 4	17 8

#### CONCLUSION

The fertilization of sea urchins' eggs which leads to an increase of four to six times its cell oxidation, is not accompanied by any increase in its catalytic activity

# STUDIES IN NUTRITION

## I THE UTILIZATION OF THE PROTEINS OF WHEAT

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(Received for publication, September 2, 1911 )

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### INTRODUCTION

For many years unlike values in nutrition have been ascribed to the proteins of animal and vegetable origin. Now that the chemical individuality and physiological specificity of the so-called proximate principles are asserting their importance,<sup>1</sup> further study of the availability of the foodstuffs seems especially

<sup>1</sup>Reference may be made to the recent researches on the amino-acid requirements of the animal organism by Abderhalden, Henriques, Michaud, (see bibliography), and others. Cf. also the studies of Hunt (Hygienic Laboratory, Public Health and Marine Hospital Service of the United States, Bull. 69, 1910), which indicate that the resistance of animals to certain poisons may vary with the character of their diet.



desirable The opinion is freely expressed that the animal proteins are far better utilized than those of plant origin, and the following statistics compiled by Atwater and Bryant<sup>1</sup> may be quoted on this point

CHARACTER OF DIET	PROTEIN UTILIZED
	<i>per cent</i>
Animal foods	97
Cereals	85
Legumes, dried	78
Vegetables	83
Fruits	85
Vegetable foods	84
Total food	92

It can scarcely be said that we are yet in a position to explain adequately the poorer utilization of the nitrogen components of certain vegetable foods Voit<sup>2</sup> and his followers<sup>3</sup> were early aware that structural peculiarities of plant products, such as cellulose walls, etc., render the proteins comparatively inaccessible to the digestive juices, thus in part explaining the possibility of poorer utilization The question of the relative availability and nutritive value of the vegetable proteins *per se* has received little attention, owing in large part to the technical difficulties in securing suitable isolated products for study

We have undertaken a detailed investigation of some of the factors involved in the digestibility and utilization of the proteins of vegetable food materials An attempt has been made to eliminate many of the unfavorable conditions or factors which attend the use of these plant products, and above all to study the nutritive value of their proteins as such Incidentally it has become necessary to devote some attention to various features of the alimentary functions, such as the origin of the feces, which have an important bearing on the interpretation of experimental results <sup>4</sup>

<sup>1</sup> Atwater and Bryant Report of the Storrs Agricultural Experiment Station, 1899, p 86

<sup>2</sup> Voit *Sitzungsberichte der Bayerischen Akademie*, 11 (4), 1869

<sup>3</sup> Cf Rubner *Zeitschrift für Biologie*, 11, p 45, 1883

<sup>4</sup> The data in this and succeeding papers of this series are taken from the dissertation of M S Fine, Yale University, 1911

## FACTORS INVOLVED

The low nitrogen content of most vegetable foods necessitates the ingestion of a relatively large volume. This *generally increased bulk of vegetable food* may of itself lead to more rapid evacuation and lessen the possibilities of digestion and absorption.

Again, in comparison with products of animal origin the vegetable foods may present an *unfavorable texture*. In older plants the cell walls may be quite tough and even supplemented with lignin. There is evidence that cellulose is not digested to any considerable extent by the higher animals,<sup>1</sup> and the vegetable membranes are not always easily permeable to the digestive juices.<sup>2</sup> It is of primary importance for digestion that the plant cells should be thoroughly disintegrated. That ordinary cooking is not sufficiently rigorous treatment to bring about complete rupture, is brought out in a subsequent paper of this series. Observations by Wicke indicate that the nitrogen utilization of diets containing much bread becomes more and more unfavorable with increase in the cellulose content. Thus, with an almost constant nitrogen intake, the nitrogen utilization was 79, 75, 63, 69, 53 per cent respectively with a cellulose concentration in the food of 0.2, 0.3, 1.1, 1.3, 1.6 per cent. This condition is probably due in great part to the incomplete rupture of the cells. Whether the cellulose *per se* exerts an unfavorable influence, that is when it cannot be accused of rendering the nutrients inaccessible to the digestive agents, is a question which has received comparatively little attention. The matter will be discussed fully in a later paper.

*Mechanical factors* may influence the rate of passage through the alimentary canal. This applies to coarse particles such as are derived from seed coats in bran. Wheat bran and similar products contain *phytin* to which a laxative action has been attributed in the case of cattle.<sup>3</sup> The fermentative development of acid and gas with the consequent stimulation of peristalsis has likewise

<sup>1</sup> Cf. Scheunert and Lötsch *Biochemische Zeitschrift*, **xx**, p. 10, 1909.  
Cf. Rubner *loc. cit.*

<sup>2</sup> Cf. Jordan, Hart, and Patten *American Journal of Physiology*, **xvi**, p. 268, 1906; Hart, McCollum and Humphrey *Wisconsin Agricultural Experiment Station, Research Bull. No. 5*, 1909, also Mendel and Underhill *American Journal of Physiology*, **xvii**, p. 75, 1905.

been pointed out in connection with utilization. For example, Menicanti and Prausnitz noted that poor utilization of bread nitrogen is accompanied by high acidity of the feces.

In addition to the preceding considerations we must ask ourselves whether the vegetable proteins by themselves exhibit any *inherent resistance to the digestive enzymes of man*<sup>1</sup>. To this question we have devoted special attention.

#### EARLIER STUDIES WITH BREAD

The actual nutritive value of bread was early investigated by Bischoff and Voit on dogs. They found the nitrogenous constituents of bread to be 80 to 84 per cent available. E. Bischoff reported a more extended study, in which the nitrogen of bread was shown to be 82 to 85 per cent utilized. When the nitrogen and starch of bread were replaced by the nitrogen of meat and pure starch, the utilization was 92 per cent, which result would lead one to believe that the low digestibility of the bread nitrogen might be attributed to the unfavorable texture.

Meyer also found that the texture plays an important rôle in the utilization. "Semmel"—white bread made of the finest flour—was 80 per cent available, while "Pumpernickel" had a digestibility of but 58 per cent. Rubner obtained results essentially the same as those reported by Meyer.

Wicke found decorticated wheat bread to be more thoroughly utilized than undecorticated, thus being in accord with Rubner's experiments, in which it was shown that the nutritive value of bread becomes lower as the bran content increases.

From the studies of Menicanti and Prausnitz it appears that the nitrogen of rye bread is less digestible (70 per cent) than that of wheat (87 per cent), while bread made of equal parts rye and wheat had a nutritive value between the two (80 to 82 per cent).

From the data<sup>2</sup> thus briefly cited, it is apparent that the nitrogenous constituents of products made of decorticated finely ground

<sup>1</sup> Cf. Moore, in *Schafer's Textbook of Physiology*, p. 441, 1898, and Hammarsten *Lehrbuch der physiologischen Chemie*, 1909.

<sup>2</sup> For other experiments, in which bread formed a larger or smaller part of the diet, see the digest by Atwater and Langworthy. Office of Experiment Stations, Bull. 45, 1897.

wheat are the most thoroughly digested,<sup>1</sup> although evidently not as completely as meat, whereas the coarse breads, made of undecorticated flours, are very poorly utilized. Between these there are all gradations, depending upon the texture. It is worthy of note that under apparently the same conditions of texture, etc., rye bread is less well utilized than wheat bread.

In the studies above reviewed the nitrogen of bread has in no case been shown to be as available to the organism as that of meat. However, it is difficult to deduce satisfactory conclusions from these experiments as there has usually been some complicating influence—bran, cellulose, acidity, etc. In the properly conducted experiment one would employ the pure protein, free from bran, starch, and cellulose, or the latter at least thoroughly disintegrated. The starch can very easily be washed out of flour, and thus a fairly pure protein preparation obtained. The resulting material—gluten—is a common commercial article. A similar product—"aleuronat"—is slightly changed gluten.

#### EARLIER STUDIES WITH ISOLATED PROTEINS

Rubner found the utilization of macaroni noodles with and without gluten to be 89 and 83 per cent respectively. However, the nitrogen intake in the former diet was twice as great as that in the latter, and there is thus the possibility that with equal nitrogen intakes the coefficients of digestibility would have been more nearly alike. In an experiment on a man Constantinidi found gluten to be 94 per cent digested, and the utilization in two experiments on a dog was 97 per cent. Potthast showed this material to be 92 per cent available, and Lusk obtained the somewhat less favorable result of 87 per cent. Kornauth found the utilization of gluten to be 91 per cent against 78 to 82 per cent for dried meat protein.

The thorough digestibility of "aleuronat" has been demonstrated by many workers, notably Bornstein, Laves, Wintgen, and Sal-kowski. In recent years gliadin, among other proteins, has been the object of study, with particular reference to its ability to main-

<sup>1</sup> Cf. Woods and Merrill. Office of Experiment Stations, Bull. 85, 1900. These authors gave the utilization of the protein of white bread as 86 per cent, being the average of thirteen experiments.

tain nitrogen equilibrium. Incidentally we may glean some data bearing upon the subject of protein utilization. Abderhalden found gliadin to be 94 to 98 per cent digested, this result being even better than the utilization of 90 per cent for horse meat. Michaud obtained coefficients of digestibility for "gliadin" of 86 to 96 per cent. Bushik and Goldhaber also worked with "gliadin" and found its utilization to be as good or better than that of meat nitrogen.

#### METHODS

The ideal method for the elucidation of the question as to the relative degree of digestibility of animal and vegetable proteins would seem to be the feeding of such mixtures of the pure foodstuffs protein, sugar and fat—free from starch and cellulose<sup>1</sup>. By this procedure one would avoid the complicating factors of excessive volume, characteristic of plant food, and the inaccessibility of the food materials due to the inclusion of these substances within the impenetrable cells. In some instances this ideal has been strictly followed, in others the cellulose was not removed, but the plant cells were thoroughly broken by heating or grinding to an impalpable powder.

In general, for the present experiments, periods of meat feeding were interposed between the experimental periods. The animals were thus kept in good condition, any disturbing influence of one diet would probably be overcome before the feeding of the next food under investigation, and finally all experimental foods were adequately controlled by the thoroughly digested meat diets. The fat content<sup>2</sup> of the meat was not determined, hence it cannot be stated to exactly what extent the caloric intakes in the different periods were comparable. One or more of the proteins of wheat supplied *all* the nitrogen of the diet. The nitrogen intakes were practically the same over long periods of time, when for any reason the nitrogen intake was changed, a preliminary period of two to three days always preceded the period of actual observation on the new nitrogen level, thus giving an opportunity for readjustment.

<sup>1</sup> For criticism of this viewpoint of Bryant and Milner *American Journal of Physiology*, v, p 34, 1903

<sup>2</sup> Arbitrarily assumed to be 10 per cent

The feces accruing from the various diets were identified by giving a capsule of lampblack or carmine<sup>1</sup> with the first meal of each period. Unless dry when collected, the feces were preserved in acidified alcohol until all the feces of the period had been assembled, whereupon they were dried on the water bath and finally ground and analyzed.<sup>2</sup>

Current discussion<sup>3</sup> would seem to indicate that this process of drying on the water bath occasions a loss of nitrogen. The error incident to this procedure, however, did not appear to us to warrant serious attention, at least not until certain details of metabolism operations, such, *e g*, as the accurate division of feces belonging to successive periods, reach a higher stage of perfection.

The feces have only occasionally been diarrhoeal and the animals have never been observed to dispose of their excrement. In many instances agar agar or bone ash or both of these indigestible materials were added to the diet. This served to offset the difficulty in obtaining satisfactory separations of successive periods due to infrequent defecations. On the other hand the objection may very properly be made that the addition of these materials unfavorably influences the protein utilization. Indeed, the experiments of Lothrop,<sup>4</sup> and also some of our own studies, amply confirm the validity of these objections. Lothrop's work shows, for example, that the addition to a meat diet of 1 gram of bone ash per kilo of body weight may almost double the nitrogen loss through the feces. However, it seems reasonable to suppose that the utilization of all diets would be similarly influenced, and thus the results for various periods would still be comparable. The actual specific influence of these indigestible materials will be discussed in a subsequent paper.

<sup>1</sup> Each capsule contained an average of 0.35 gram carmine=0.02 gram nitrogen—a negligible quantity.

<sup>2</sup> The weights of the feces and the percentage nitrogen composition are based upon the air-dried specimens. The comparisons of these values which will be frequently made throughout this work are, nevertheless, considered permissible since variations due to this cause would undoubtedly be smaller than incidental variations from other causes.

<sup>3</sup> Cf. Howe, Rutherford, and Hawk. *Journal of the American Chemical Society*, xxxii, p. 1683, 1910. For the literature see Emmet and Grindley *ibid.*, xxxi, p. 569, 1909.

<sup>4</sup> Lothrop. *American Journal of Physiology*, xxiv, p. 297, 1909.

## EXPERIMENTAL PART

*Products Employed*

I "*Gludin*,"<sup>1</sup> a commercial preparation This material is a slightly yellowish and tasteless white powder, which, according to Bergell,<sup>2</sup> and Thiemer<sup>3</sup> is prepared from wheat flour by a process of washing and centrifuging

II *Gluten*,<sup>4</sup> a commercial preparation manufactured by the Kellogg Food Company, of Battle Creek, Mich It consisted of thin, flat, yellow scales, approximately  $\frac{1}{16}$  inch in diameter

III *Glutenin*<sup>5</sup> The specimen used in these trials was prepared as follows Wheat flour was washed thoroughly with water to remove the starch The resulting gluten was then extracted four times with 70 per cent alcohol, *et cetera*, until the extracts were colorless, and practically all gliadin was removed The crude glutenin thus obtained was dried, finely ground, extracted once with ether, and finally ground to an impalpable powder It was not dissolved in alkali and reprecipitated with acid, and hence still contained practically all the cellulose of the original wheat flour On the other hand, the possibility of the protein being changed by solution in alkali was avoided

IV *Gliadin*<sup>6</sup> The 70 per cent alcoholic extract obtained in preparing glutenin, as described above, was concentrated to a thick syrup, and precipitated by pouring into water containing a little salt This glue-like material was dissolved in alcohol, whose final strength was 70 per cent, and this time precipitated by pouring into 95 per cent alcohol The material thus obtained was dried with alcohol and ether and ground to an impalpable powder

Dr T B Osborne very kindly supplied us with both glutenin and gliadin in sufficient quantities

<sup>1</sup> Obtained from Menley and James, New York City The material contained 14.5 per cent nitrogen, and did not give a starch reaction Considerable of this preparation dissolved in warm 70 per cent alcohol, reprecipitating on pouring into cold water—a characteristic behavior of gliadin

<sup>2</sup> Bergell *Medizinische Klinik*, No 41, p 1042, 1905

<sup>3</sup> Thiemer *Wiener medizinische Presse*, No 47, p 2431, 1906

<sup>4</sup> This material was very kindly furnished by Dr Kellogg It contained 14 per cent nitrogen

<sup>5</sup> For description of glutenin and gliadin, see T B Osborne "Die Pflanzenproteine," *Ergebnisse der Physiologie*, v, p 47, 1910

<sup>6</sup> Cf preceding footnote

## Metabolism Experiments

"GLIDIN"—Tables 1 to 3 Detailed information on the nature of the food ingredients may be obtained from the tables In the periods of meat feeding, the mixture of cane sugar, lard, agar and bone ash was heated on the water bath, till the lard had melted,

TABLE 1  
"Glidin" with Agar and Bone Ash

SUBJECT DOG 5 Weight at beginning 5.4 Kg Weight at end 5.2 Kg	PERIOD III (4 days) Meat Feeding	PERIOD IV (4 days) Glidin Feeding	PERIOD V (5 days) Glidin Feeding	PERIOD VI (4 days) Meat Feeding
	grams	grams	grams	grams
Composition of daily diet	Meat 150	"Glidin" 34	"Glidin" 34	Meat 150
	Sugar 20	Sugar 20	Sugar 20	Sugar 20
	Lard 20	Lard 30	Lard 30	Lard 20
	Agar 3	Agar 3	Agar 3	Agar 3
	Bone Ash 7	Bone Ash 7	Bone Ash 7	Bone Ash 7
	Water 100	Water 200	Water 200	Water 100
	Estimated calories 520	Estimated calories 470	Estimated calories 470	Estimated calories 520
	Daily Averages	Daily Averages	Daily Averages	Daily Averages
<i>Nitrogen output</i>				
Urine nitrogen, gm	3.80	4.63	4.85	3.71
Total nitrogen, gm	4.16	4.95	5.20	4.11
Nitrogen in food, gm	4.90	4.93	4.93	4.93
Nitrogen balance, gm	+0.74	-0.02	-0.27	+0.82
<i>Feces</i>				
Weight air dry, gm	14.5	15.5	15.4	15.5
Nitrogen, gm	0.36	0.32	0.35	0.40
Nitrogen, per cent	2.48	2.03	2.25	2.60
Nitrogen utilization, per cent	92.7	93.6	93.0	91.8

whereupon the meat<sup>1</sup> and water were added,<sup>2</sup> the whole being thoroughly mixed In the "glidin" periods, the food mixture was warmed on the water bath the day before feeding, the water thor-

<sup>1</sup> Preserved frozen, according to the method of Gies

<sup>2</sup> Just before feeding



oughly incorporated, and the whole allowed to stand over night, thus giving ample time for "hydration"<sup>1</sup> of the material

Such food mixtures were fed for periods of 3 to 5 days to three small bitches. The food was disposed of in one meal at 9 00 to 9 45 each morning

TABLE 2

*"Ghlin" with Agar and Bone Ash*

SUBJECT DOG 6 Weight at beginning, 5.0 Kg Weight at end, 4.9 Kg		PERIOD IV (5 days) Meat Feeding	PERIOD V (5 days) Ghlin Feeding	PERIOD VI (4 days) Meat Feeding
Composition of daily diet		grams	grams	grams
		Meat 150	"Ghlin" 34	Meat 150
		Sugar 20	Sugar 20	Sugar 20
		Lard 20	Lard 30	Lard 20
		Agar 3	Agar 3	Agar 3
		Bone Ash 7	Bone Ash 7	Bone Ash 7
		Water 100	Water 200	Water 100
		Estimated calories 520	Estimated calories 470	Estimated calories 520
		Daily Averages	Daily Averages	Daily Averages
<i>Nitrogen output</i>				
Urine nitrogen, gm		3.70	4.83	4.13
Total nitrogen, gm		4.06	5.14	4.48
Nitrogen in food, gm		4.93	4.93	4.93
Nitrogen balance, gm		+0.87	-0.21	+0.45
<i>Feces</i>				
Weight air dry, gm		13.6	13.6	14.5
Nitrogen, gm		0.36	0.31	0.35
Nitrogen, per cent		2.64	2.31	2.42
Nitrogen, utilization, per cent		92.7	93.6	92.9

From an examination of the tables, it will be observed that the data without exception point to the fact that "ghlin" is as thoroughly utilized as meat under identical conditions

<sup>1</sup> When first added to the food mixture there appeared to be little tendency for the water to be absorbed. On standing a few hours, however, and especially the next morning, a thick thoroughly hydrated mush resulted

TABLE 3

*"Glidin" with Agar and Bone Ash*

SUBJECT, DOG 7 Weight 4.9 Kg	PERIOD III (5 days) Meat Feeding	PERIOD IV (5 days) Glidin Feeding	PERIOD V (3 days*) Meat Feeding
	grams	grams	grams
Composition of daily diet	Meat 100	"Glidin" 23	Meat 100
	Sugar 20	Sugar 20	Sugar 20
	Lard 25	Lard 30	Lard 20
	Agar 3	Agar 3	Agar 3
	Bone Ash 7	Bone Ash 7	Bone Ash 7
	Water 100	Water 175	Water 100
	Estimated calories 480	Estimated calories 430	Estimated calories 430
	Daily Averages	Daily Averages	Daily Averages
Nitrogen output			
Urine nitrogen, gm	2.58	3.21	2.63
Total nitrogen, gm	2.81	3.48	2.89
Nitrogen in food, gm	3.29	3.29	3.29
Nitrogen balance, gm	+0.48	-0.19	+0.40
Feces			
Weight air dry, gm	12.8	11.0	12.7
Nitrogen, gm	0.23	0.27	0.26
Nitrogen, per cent	1.81	2.43	2.04
Nitrogen utilization, per cent	93.0	91.9	92.1

COMMERCIAL GLUTEN—Tables 4 to 9. In Tables 4 to 6 are recorded experiments wherein the utilization of gluten, fed with agar and bone ash, is compared with meat diets in which identical additions of these indigestible materials were made. Data on the comparative utilization of gluten and meat, where no agar or bone ash was employed, are reported in Tables 7 to 9.

An examination of these tables will readily convince one that the present sample of *commercial gluten* is as thoroughly utilized as meat.

One might take exception to this generalization, observing that the persistently high nitrogen content of the gluten-feces, as compared with that of the corresponding meat-feces, indicates that a portion of the gluten had been lost through the feces. Moreover,

careful scrutiny will disclose the fact that the utilization of the gluten is consistently—if only slightly—lower than that of meat. This criticism is indeed valid. However, had the gluten been finely divided, objections of the above nature, *which in any case are concerned with small differences*, would without doubt be untenable.

TABLE 4

*Gluten with Agar and Bone Ash*

SUBJECT, DOG 5 Weight at beginning, 5.2 Kg    Weight at end 5.2 Kg		PERIOD VIII (5 days) Meat Feeding	PERIOD IX* (6 days) Gluten Feeding
		grams	grams
Composition of daily diet	Meat	150	Gluten 36
	Sugar	20	Sugar 20
	Lard	20	Lard 30
	Agar	3	Agar 3
	Bone Ash	7	Bone Ash 7
	Water	100	Water 200
	Estimated calories	520	Estimated calories 480
		Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm		4.09	4.78
Total nitrogen, gm		4.44	5.22
Nitrogen in food, gm		4.80	4.90
Nitrogen balance, gm		+0.36	-0.32
<i>Feces</i>			
Weight air dry, gm		15.0	14.5
Nitrogen, gm		0.35	0.44
Nitrogen, per cent		2.32	3.05
Nitrogen utilization, per cent		92.8	91.0

\* On last two days of period, about half the food was forced

TABLE 5  
*Gluten with Agar and Bone Ash*

SUBJECT DOG 6 Weight at beginning 47 Kg Weight at end 45 Kg		PERIOD VIII (5 days) Meat Feeding	PERIOD IX (5 days) Gluten Feeding
Composition of daily diet		grams	grams
		Meat 150	Gluten 36
		Sugar 20	Sugar 20
		Lard 20	Lard 30
		Agar 3	Agar 3
		Bone Ash 7	Bone Ash 7
		Water 100	Water 200
		Estimated calories 520	Estimated calories 480
Nitrogen output		Daily Averages	Daily Averages
Urine nitrogen, gm		4 23	4 81
Total nitrogen, gm		4 62	5 24
Nitrogen in food, gm		4 80	4 90
Nitrogen balance, gm		+0 18	-0 34
<i>Feces</i>			
Weight air dry, gm		15 0	14 4
Nitrogen, gm		0 39	0 43
Nitrogen, per cent		2 59	3 00
Nitrogen utilization, per cent		91 9	91 2

TABLE 6  
*Gluten with Agar and Bone Ash*

SUBJECT DOG 7 Weight at beginning 46 Kg Weight at end 46 Kg		PERIOD VII (5 days) Meat Feeding	PERIOD VIII (5 days) Gluten Feeding
Composition of daily diet		grams	grams
		Meat 100	Gluten 24
		Sugar 20	Sugar 20
		Lard 20	Lard 30
		Agar 3	Agar 3
		Bone Ash 7	Bone Ash 7
		Water 100	Water 175
		Estimated calories 430	Estimated calories 430
Nitrogen output		Daily Averages	Daily Averages
Urine nitrogen, gm		2 55	3 24
Total nitrogen, gm		2 79	3 52
Nitrogen in food, gm		3 20	3 27
Nitrogen balance, gm		+0 41	-0 25
<i>Feces</i>			
Weight air dry, gm		12 8	12 2
Nitrogen, gm		0 24	0 28
Nitrogen, per cent		1 86	2 31
Nitrogen utilization per cent		92 6	91 4

TABLE 7

*Gluten without Agar or Bone Ash*

SUBJECT DOG 5 Weight at beginning, 5.0 Kg    Weight at end 5.8 Kg		PERIOD XX (4 days) Meat Feeding	PERIOD XXII* (5 days) Gluten Feeding
Composition of daily diet		grams	grams
		Meat 150	Gluten 34
		Sugar 25	Sugar 25
		Lard 20	Lard 25
		Water 100	Water 225
		Estimated calories 530	Estimated calories 440
<i>Nitrogen output</i>		Daily Averages	Daily Averages
Urine nitrogen, gm		4.08	4.83
Total nitrogen, gm		4.30	5.05
Nitrogen in food, gm		4.59	4.77
Nitrogen balance, gm		+0.29	-0.28
<i>Feces</i>			
Weight air dry, gm		4.5	2.8
Nitrogen, gm		0.22	0.22
Nitrogen, per cent		4.95	7.68
Nitrogen utilization, per cent		95.2	95.5

\* About half of the food forced each day

TABLE 8

*Gluten, without Agar or Bone Ash*

SUBJECT DOG 6 Weight at beginning, 6.1 Kg    Weight at end 6.2 Kg		PERIOD XXI (4 days) Meat Feeding	PERIOD XXIII (5 days) Gluten Feeding
Composition of daily diet		grams	grams
		Meat, 150	Gluten 34
		Sugar 25	Sugar 25
		Lard 20	Lard 25
		Water 100	Water 225
		Estimated calories 530	Estimated calories 440
<i>Nitrogen output</i>		Daily Averages	Daily Averages
Urine nitrogen, gm		3.55	4.92
Total nitrogen, gm		3.77	5.17
Nitrogen in food, gm		4.59	4.77
Nitrogen balance, gm		+0.82	-0.40
<i>Feces</i>			
Weight air dry, gm		3.5	3.6
Nitrogen, gm		0.22	0.25
Nitrogen, per cent		6.34	6.93
Nitrogen utilization, per cent		95.2	94.8

TABLE 9

*Gluten without Agar or Bone Ash*

SUBJECT DOG 7 Weight at beginning 59 Kg    Weight at end 63 Kg		PERIOD XX (4 days) Meat Feeding	PERIOD XXII (5 days) Gluten Feeding
		grams	grams
Composition of daily diet	Meat	150	Gluten 34
	Sugar	25	Sugar 25
	Lard	20	Lard 25
	Water	100	Water 225
	Estimated calories	530	Estimated calories 440
		Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm		3 55	4 68
Total nitrogen, gm		3 74	4 81
Nitrogen in food, gm		4 59	4 77
Nitrogen balance, gm		+0 85	-0 04
<i>Feces</i>			
Weight air dry, gm		3 2	1 8
Nitrogen, gm		0 19	0 13
Nitrogen per cent		5 93	7 40
Nitrogen utilization, per cent		95 8	97 2

GLUTENIN—Tables 10, 11    *Man, Table 10*    The subject of this experiment, M S F, was 23 years of age, about 57 5 kilos in weight. Throughout the experiment he was engaged in the routine connected with such work. The general plan of the experiment was as follows. A preliminary period of three days was obtained, during which the body was enabled to attain nitrogen equilibrium and to adjust itself to the experimental conditions. As will be noted from the accompanying table, during the preliminary, fore, and after periods, a varied mixed diet was consumed, and during the experimental period, the meat, nut butter and part of the egg were replaced by glutenin.

*Character of the Diet*

	PRELIMINARY, FORE AND AFTER PERIODS	EXPERIMENTAL PERIOD
	Daily Averages	Daily Averages
	<i>Gm</i>	<i>Gm</i>
Cereal	20	20
Cracker	60	60
Egg	110	50
Pine nut butter	50	
Meat	110	
Glutenin		62
Potato	110	110
Banana	140	140
Apple	270	250
Orange	160	180
Milk	40	40
Sugar	110	180
Butter	40	80
Cereal coffee, tea	600	600

TABLE 10

*Glutenin*

SUBJECT, MAN Weight at beginning, 57.8 Kg Weight at end, 57.6 Kg	PERIOD I (6 days) Mixed Diet	PERIOD II (4 days) Glutenin	PERIOD III (4 days) Mixed Diet
Composition of daily diet	Meat, eggs, nut butter, cereal, potato, fruit, etc	Glutenin cereal, potato, fruit, etc 67.2 per cent of the total nitrogen fur- nished by glutenin	Meat, eggs, nut butter, cereal, potato, fruit, etc
	Estimated calories 2400	Estimated calories 2500	Estimated calories 2600
	Daily Averages	Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm	9.85	10.80	9.98
Total nitrogen, gm	11.10	12.11	11.36
Nitrogen in food, gm	11.30	11.12	11.15
Nitrogen balance, gm	+0.25	-0.99	-0.21
<i>Feces</i>			
Weight air dry, gm	23.0	22.1	24.7
Nitrogen, gm	1.25	1.31	1.38
Nitrogen, per cent	5.45	5.92	5.63
Nitrogen utilization, per cent	89.0	88.2	87.6

These diets furnished about 11.2 grams nitrogen and 2,500 calories per day, during the experimental period 67 per cent of the nitrogen was supplied by the glutenin.

During the evening of the third day of the experimental period, slight indigestion was manifest, which continued to the following morning, when 100 cc of 0.2 per cent HCl were taken.<sup>1</sup> The glutenin had a peculiar ether-alcohol odor which could not be removed by drying at 130° C for eight consecutive hours. This odor was very disagreeable and nauseating and could not be covered by mixing with other foods. It was found that mixing the glutenin with egg and potato, and frying this mixture, was practically the only way in which the glutenin could be consumed in any quantity. Under such circumstances it is probable that the psychic secretion was a negligible quantity and this may account for the symptoms of indigestion noted.

With the exception of the feeling of nausea attending the meals of the four days of glutenin feeding, the subject felt perfectly well. Throughout the experiment defecation was accomplished regularly every morning, the volume of feces being apparently alike from day to day. The feces were of semi-solid consistency, never well formed. There was, however, nothing approaching diarrhoea, throughout the experiment, except on the last day of glutenin feeding.

In spite of the nausea with its possible secretory consequences, *the glutenin appeared to be as well digested as the materials which it replaced in the control diets.* It should be observed, however, that about 25 per cent of the nitrogen of the control diets was furnished by nut butter, and, according to Jaffa,<sup>2</sup> the nitrogen of nuts is in general only about 75 per cent utilized. Hence, in order to show the readily digestible nature of glutenin, the coefficient of digestibility in the experimental period should have been somewhat greater than those for the control periods. Nevertheless, it is significant to note that in two experiments on the same subject, where mixed diets were employed, which contained no nut preparations but were otherwise similar, the utilization was 88 and 86 per cent, respectively.

<sup>1</sup> This affects one day out of four, and since the HCl gave no noticeable relief, the influence of the acid even in this day was probably very small.

Jaffa. Office of Experiment Stations, Bull. 132, p. 69, 1903.



TABLE 11

*Glutenin with Agar and Nutrient "Salts"*

SUNJIC, DOG 4 Weight at beginning, 5.1 Kg Weight at end, 5.0 Kg	PERIOD I (5 days) Meat Feeding	PERIOD II* (5 days) Glutenin Feeding	PERIOD III (3 days) Meat Feeding
Composition of daily diet	<i>grams</i>	<i>grams</i>	<i>grams</i>
	Meat 150	Glutenin 43	Meat 150
	Sugar 15	Sugar 25	Sugar 25
	Starch 5	Starch 5	Starch 5
	Lard 20	Lard 30	Lard 20
	Agar 8	Agar 8	Agar 8
	Salts 2	Salts 4	Salts 4
	Water 200	Water 300	Water 200
	Estimated calories 520	Estimated calories 520	Estimated calories 560
	Daily Averages	Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm	4.71	4.71	4.18
Total nitrogen, gm	5.04	5.01	4.48
Nitrogen in food, gm	5.18	5.18	5.18
Nitrogen balance, gm	+0.14	+0.17	+0.70
<i>Feces</i>			
Weight air dry, gm	13.6	15.0	12.7
Nitrogen, gm	0.33	0.30	0.31
Nitrogen, per cent	2.40	1.99	2.42
Nitrogen utilization, per cent	93.7	94.3	94.1

\* About half of the food forced each day

For mixed diets on man 93 per cent is given as the average coefficient of digestibility by Atwater and Bryant,<sup>1</sup> being considerably higher than the coefficients obtained in our experiments. The difference may be accounted for by the presence in the diets of relatively large amounts of fruits and vegetables, which contain considerable indigestible material.<sup>2</sup>

*Dog, Table 11* The plan of the experiment did not differ essentially from those already described. The character of the indi-

<sup>1</sup> See footnote, 1 p. 304<sup>2</sup> Cf. Bryant and Milner *American Journal of Physiology*, x, p. 96, 1903

gestible materials was somewhat different, larger amounts of agar being used, without bone ash, the latter being replaced by a salt mixture<sup>1</sup>. Despite the necessity of forced feeding, *the glutenin was quite as thoroughly utilized as meat fed under similar experimental conditions*

**GLIADIN**—Tables 12, 13 *Man, Table 12* The plan of the experiment was essentially that outlined under glutenin, except that slightly more meat was eaten in the control periods, and in the gliadin period the egg, meat and nut butter were *completely* replaced by gliadin, which supplied 85 per cent of the total nitrogen. The gliadin when mixed with water forms a veritable glue, which cannot possibly be eaten. This glue-like consistency was avoided by mixing with cornstarch, salt, sugar, and baking powder, which mixture was finally baked. Even in this condition, the material soon evoked nausea which, on the last day of the period, made a discontinuance necessary. Aside from the nausea attending the meals of the gliadin period, the subject felt in excellent condition throughout the experiment, diarrhoea and symptoms of indigestion being entirely absent.

From Table 12, it is evident that *gliadin is as thoroughly utilized as the materials of the control periods, which it replaced*

The salts of the baking powder seemed to have no appreciable influence. The apparently low digestibility of the mixed diets has already been discussed under glutenin.

*Dog, Table 13* The plan of this experiment was exactly the same as that described on page 320 under the glutenin experiment on a dog. The food mixture was like so much glue, but the animal

<sup>1</sup> With one or two modifications this is the salt mixture proposed by Röhmnn (*Allgemeine medizinische Central-Zeitung*, No 9, 1903). It consisted of the following ingredients

	Gram
Calcium phosphate	10
Acid potassium phosphate	37
Sodium chloride	20
Sodium citrate	15
Magnesium citrate	8
Calcium lactate	8
Ferric citrate	2

TABLE 12

*Ghadin*

SUBJECT MAN Weight at beginning 57.0 Kg Weight at end 57.6 Kg	PERIOD I (5 days) Mixed Diet	PERIOD II (4 days) Ghadin	PERIOD III (4 days) Mixed Diet
Composition of daily diet	Meat, eggs, nut butter, potatoes, fruit, etc	Ghadin, potatoes, fruit, etc 84.8 per cent of the total ni- trogen sup- plied by glia- din	Meat, eggs, nut butter, potatoes, fruit, etc
	Estimated calories 2600	Estimated calories 2800	Estimated calories 2900
	Daily Averages	Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm	10.72	11.56	10.10
Total nitrogen, gm	12.13	12.83	11.38
Nitrogen in food, gm	12.72	12.02	12.68
Nitrogen balance, gm	+0.60	-0.80	+1.30
<i>Feces</i>			
Weight air dry, gm	23.6	22.5	26.8
Nitrogen, gm	1.41	1.27	1.53
Nitrogen, per cent	5.96	5.53	5.74
Nitrogen utilization, per cent	89.0	89.4	87.9

ate it with apparent relish. From Table 13 *the glutenin appears to have been quite thoroughly utilized*.

Abderhalden obtained plus balances with ghadin, but notes that his preparations contained 0.35 per cent lysine—an amino-acid not found in pure ghadin. He infers further that it is not known whether really pure ghadin can maintain nitrogen equilibrium. We are unable to state whether or not the ghadin employed in our experiments was free from lysine, but *the negative nitrogen balances, even with large intakes, are significant*. Henriques obtained plus balances with ghadin in rats, but Abderhalden questions the purity of his preparations.<sup>1</sup>

<sup>1</sup> Osborne and Mendel (*Feeding Experiments with Isolated Food-Substances*, Carnegie Institution of Washington, Publication No. 156 p. 21,

TABLE 13

*Gladin with Agar and Nutrient "Salts"*

SUBJECT DOG 4 Weight at beginning 49 Kg Weight at end 49 Kg	PERIOD III (3 days) Meat Feeding	PERIOD IV (3 days) Gladin Feeding	PERIOD V (4 days) Meat Feeding
Composition of daily diet	Meat 150 <sup>gram</sup>	Gladin 32 <sup>gram</sup>	Meat 150 <sup>gram</sup>
	Sugar 25	Sugar 25	Sugar 25
	Starch 5	Starch 5	Starch 5
	Lard 20	Lard 30	Lard 20
	Agar 8	Agar 8	Bone Ash 10
	Salts 4	Salts 4	Water 200
	Water 200	Water 250	
	Estimated calories 560	Estimated calories 520	Estimated calories 560
	Daily Averages	Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm	4 18	5 09	4 24
Total nitrogen, gm	4 48	5 37	4 50
Nitrogen in food, gm	5 18	5 10	5 40
Nitrogen balance, gm	+0 70	-0 27	+0 90
<i>Feces</i>			
Weight air dry, gm	12 7	13 4	15 0
Nitrogen, gm	0 31	0 28	0 27
Nitrogen, per cent	2 42	2 07	1 79
Nitrogen utilization, per cent	94 1	94 5	95 0

It is difficult at present to account for the persistent negative balances with the other protein preparations. Michaud did indeed obtain minus balances with "gladin," but his nitrogen intakes were very small, while those in our experiments were relatively large.

1911) state that in rat experiments at least 10 per cent of the excreted nitrogen may be lost in connection with the difficult manipulation attending metabolism experiments with small animals. This condition may in part account for the positive balances obtained by Henriques.

## Nitrogen Balances

TABLE 14

*Average Daily Nitrogen Balances for the Wheat Proteins and Corresponding Values for Meat*

SUBJECT	TABLET	"GLIDIN"	'GLUTEN'	GLUTENIN	GLIADIN	MEAT
Dog 5	1	-0 02, -0 27				+0 74, +0 82
Dog 6	2	-0 21				+0 87, +0 45
Dog 7	3	-0 19				+0 48, +0 40
Dog 5	4		-0 32			+0 36
Dog 6	5		-0 34			+0 18
Dog 7	6		-0 25			+0 41
Dog 5	7		-0 28			+0 29
Dog 6	8		-0 40			+0 82
Dog 7	9		-0 04			+0 85
Man	10			-0 99		+0 25, -0 21
Dog 4	11			+0 17		+0 14, +0 70
Man	12				-0 80	+0 60, +1 30
Dog 4	13				-0 27	+0 70, +0 90

## SUMMARY

The problems associated with the utilization of food products of plant origin have been reviewed as an introduction to a series of experimental studies on the nutritive value of vegetable proteins. It is pointed out that two distinct questions must be considered, namely (1) the availability of the products existing more or less in their native condition, with accompanying structural elements, as in bread, (2) the specific utilization of the proteins themselves. The latter aspect is the one which primarily calls for further investigation.

In our feeding experiments an attempt has been made to control the extraneous factors as far as possible, by improving the texture and mechanical condition of the crude products, or purifying the individual proteins. The present paper deals with wheat. The experimental trials on man and dogs indicate that "*glidin*," *gluten*, and the two characteristic proteins of wheat, *gliadin* and *glutenin*, are as thoroughly utilized as the nitrogenous components of fresh meat.

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# ON THE MAXIMUM PRODUCTION OF HIPPURIC ACID IN ANIMALS WITH CONSIDERATION OF THE ORIGIN OF GLYCOCOLL IN THE ANIMAL BODY

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(Received for publication, September 9, 1911)

## INTRODUCTION

Parker and Lusk<sup>1</sup> fed lithium benzoate to rabbits, with the object of ascertaining how much glycocoll the animals may eliminate in the formation of hippuric acid. They also studied the relationship between the total nitrogen in the urine and the glycocoll nitrogen eliminated as hippuric acid  $\frac{H A N}{N}$ . They found that for every 100 grams of total nitrogen excreted in the urine, an average of 4 grams of nitrogen was eliminated in the glycocoll radical of the hippuric acid. After administering the first large dose of lithium benzoate to the animals, they invariably obtained a much higher  $\frac{H A N}{N}$  ratio, 9.01 per cent in experiment III, 7.14 per cent in experiment IV and 7.87 per cent in experiment VI. They regarded this as a "sweeping out of the surplus glycocoll" (in the sense of Wiener<sup>2</sup>).

Since then Wiechowski<sup>3</sup> and Magnus-Levy<sup>4</sup> simultaneously, but independently, have studied the same problem. Wiechowski obtained  $\frac{H A N}{N}$  ratios of 45.4, 55, 50, and in one case even as

<sup>1</sup> *Amer Journ of Physiol*, III, p 472, 1900

<sup>2</sup> *Arch f exp Path u Pharm*, VI, p 313, 1898

<sup>3</sup> *Beitr z chem Path u Physiol*, VII, p 204, 1906

<sup>4</sup> *Biochem Zeitschr*, VI, p 523, 1907



much as 64.3 per cent. Magnus-Levy found in his rabbits a maximal ratio of 25 and 28 per cent, in his sheep 27.8 per cent.

The various forms of animal protein, with the exception of casein which on analysis yields no glycocoll, and gelatin which yields 16 per cent, contain on an average 4 per cent of glycocoll. Since the nitrogen content of protein is 16 per cent and that of glycocoll 18.7 per cent, 100 grams of protein nitrogen therefore contain

$$\frac{100 \times 18.7 \times 4}{16} = 47 \text{ grams of preformed glycocoll nitrogen}$$

Magnus-Levy found that after feeding large doses of benzoic acid to rabbits and sheep, they were able to yield six times that amount of glycocoll. Wiechowski's rabbits yielded almost fourteen times that amount. These findings stand in direct contradiction to the conclusions arrived at by Parker and Lusk. To investigate the cause of these discrepancies, Professor Lusk kindly suggested that I continue the study of this problem. It may be added here, that Parker and Lusk were influenced by the rather inaccurate statement of Wiener, that 1.7 grams of benzoic acid per kilogram of body weight administered to rabbits was fatally toxic.

#### METHODS OF EXPERIMENTATION

The animals were kept in metabolism cages. The urine was collected by catheter and separated into twenty-four-hour periods. Sodium benzoate dissolved in water was given *per os* by means of a stomach tube. The daily dose was always divided into three to five doses, given five to three hours apart. The animals were catheterized before the stomach tube was introduced, so as to prevent any accidental loss of urine.

The total nitrogen was determined in duplicate by the ordinary Kjeldahl method. The hippuric acid, also in duplicate, was determined by the method proposed by Dakin.<sup>1</sup> One-quarter of the total quantity of urine was evaporated on the water bath to about 75 cc. After transferring to a small Erlenmeyer flask, 15 cc. of a 50 per cent solution of phosphoric acid was added. The mixture was then cooled in running tap water. The greater amount of hippuric acid then crystallized out. The solution was filtered and the crystals washed with a small quantity of ice water (Filtrate A). The crystals were then dissolved in a rather large quantity of warm water, transferred to a separatory funnel, and shaken with about

<sup>1</sup> This *Journal*, vii, p. 103, 1910

50 to 100 cc of a mixture of 2 parts of benzol and 1 part of alcohol-free ether. This removed any free benzoic acid which might have come down with the hippuric acid. After drawing off the water solution of the hippuric acid, it was slowly evaporated and crystallized. The crystals were separated by filtration and washed with a small quantity of cold water (Filtrate B). The crystals were dried and weighed in the usual manner (Gooch crucibles were found to be very convenient receptacles for the crystals).

To Filtrate A about 75 per cent of its weight of ammonium sulphate<sup>1</sup> was added. It was then placed in a continuous extraction apparatus and extracted with ethyl acetate for a period of eight to ten hours.

The ethyl acetate was then drawn off into a separatory funnel and shaken with a small quantity of a saturated sodium chloride solution. This removed any urea which might have been extracted by the ethyl acetate. The salt solution was then drawn off, and the ethyl acetate washed with a few cubic centimeters of distilled water to remove the crystals of sodium chloride which remained attached to the funnel. The salt solution, which had been drawn off, was then shaken with some ethyl acetate, which was finally added to the original.

The combined ethyl acetate solutions were then distilled *in vacuo* and the residue dissolved in hot water. This was boiled with a small quantity of blood charcoal to decolorize it, filtered, and the charcoal washed with a small quantity of boiling hot water. After the filtrate had cooled, it was placed in a separatory funnel and shaken four to five times with about 100 cc of the benzol and ether mixture. The aqueous solution of hippuric acid was then drawn off, and Filtrate B added to it. The combined solution was allowed to evaporate slowly and the hippuric acid which crystallized was purified, dried and weighed in the usual manner.

The sum of all the crystals obtained, multiplied by four, gave the value of hippuric acid excreted during the course of the period.

#### CONTROL OF THE METHOD

To 100 cc of normal human urine 1 gram of hippuric acid (Kahlbaum) was added. Recovered from the first crystallization, 0.8034, from the second, 0.1173, from the third, 0.0580 gram. Total = 0.9787 gram, or 97.87 per cent.

The melting point of the crystals was determined in every case. It was found to be between 182° and 187° C (uncorrected). The nitrogen content of the crystals was found to be between 7.94 and 7.99 per cent.

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<sup>1</sup> The ammonium sulphate serves a double purpose. First, it increases the rate of extraction of the hippuric acid. Second, it prevents the solution of the ethyl acetate by the water, which would obliterate the line of demarcation between the watery solution and the ethyl acetate.

Not all the hippuric acid crystallized out from the mother liquid with the first crystallization. Two, and sometimes three crystallizations were required to bring them all down.

The duplicates of the hippuric acid determinations agreed within 0.3 per cent

#### EXPERIMENT I

A goat weighing 42.3 Kg. was employed. She was brought to the laboratory about a week prior to the commencement of the experiment and was placed in a metabolism cage. During the foreperiod, which lasted for three days, the goat was fed on hay, cornmeal and oats. Only the total nitrogen eliminated in the urine was studied.

During the benzoate period the diet could not be regulated very well. She was fed on hay, bread, carrots, cabbage and milk, the daily quantity of which is recorded in Table I. During the periods VI, VII and VIII, she did not take any food.

During the first day of the benzoate period, the animal received 10 grams of sodium benzoate, the second, 20 grams, the third, 20 grams, the fourth, 25 grams, the fifth, 10 grams, the sixth, 30 grams, the seventh, 30 grams. On the eighth day she also received 30 grams, but all in one dose. At the end of that day the animal was still in very good condition and showed no signs of intoxication.

After sixteen days intermission an attempt was made to discover a dose which would produce toxic symptoms in the animal. To this end the animal was given 50 grams of sodium benzoate in three equal portions during the course of the day (period IX). On the following morning, distinct signs of intoxication were present. The animal was exceedingly restless. She ran about the cage, striking her horns violently against the walls. She partook of neither food nor drink. When allowed to walk out of the cage, she did not walk in a straight line, but in a zigzag manner. When she struck the wall of the room, she did not turn backwards, but kept on pushing in the same direction. In walking she did not avoid any object placed in her way. She gave the impression of having lost the power of seeing. It is to be regretted that the reflex of the eye towards light was not tested. These symptoms persisted until the following morning, when the animal was seized by an attack of tonic and clonic convulsions in which she died.

Autopsy revealed no macroscopical changes in the organs. She was very much emaciated. On section the kidneys showed slight parenchymatous tubular degeneration.



*Discussion of Results of Experiment I* The results obtained from the first experiment are summarized in Table I. It shows that the animal was able to eliminate in its urine a good deal more glycocholic acid than is found preformed in the proteins of its tissues. The hippuric acid formation, *i. e.*, the glycocholic acid elimination, does not depend upon the amount of the protein catabolized, but within certain limits, runs parallel to the amount of benzoic acid fed. In period VI it reached its maximum, when 38.4 per cent of the total nitrogen was eliminated as glycocholic acid nitrogen in the hippuric acid.

Where do the enormous quantities of glycocholic acid originate? There are a number of possibilities which have to be taken into consideration.

It might be argued, since herbivorous animals have the power of eliminating more glycocholic acid than do carnivorous animals, when given a similar quantity of benzoate, that the source of the glycocholic acid found in the system lies in the different vegetable foods which are known to contain rather large quantities of free amino bodies. The results of experiment II, however, show that this assumption is not well founded.

#### EXPERIMENT II

A fifteen day old calf, which had not yet received any other form of food but its mother's milk, the protein of which contains practically no glycocholic acid, was placed in a metabolism cage, and its urine was collected for about twelve hours. On the following morning, the calf received 8 grams of sodium benzoate *per os*. Its urine was then collected for six hours.<sup>1</sup>

TABLE II

*Calf*

DATE, 1910	NUMBER OF HOURS	TOTAL NITROGEN	HIPPURIC ACID	HIPPURIC ACID NITROGEN	HIPPURIC ACID N TOTAL N	A BENZOIC ACID FED	B BENZOIC ACID EX- CRETED AS HIP- PURIC ACID	A B
		grams	grams	gram	per cent	grams	grams	per cent
March 19	12	2.43						
March 20	6	2.17	6.827	0.539	24.8	6.78	4.66	68.6

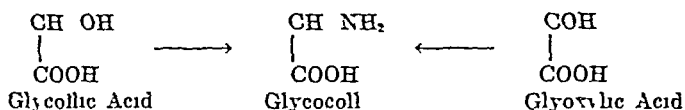
<sup>1</sup> The catheter was not used. Hence the results are only approximate.

We see from Table II that the suckling calf yielded hippuric acid as readily as would an adult goat. The glycoll in the hippuric acid could not have come from any other source than from a specialized protein metabolism.

Knoop<sup>1</sup> fed  $\alpha$ -ketonic acids to animals and obtained the corresponding amino-acids, showing that amination of non-nitrogenous substances can take place in the animal organism.



$\alpha$ -Ketonic acids, together with the corresponding oxy-acids, can be found in the organism as a result of the oxidation of the  $\alpha$ -amino bodies, or from the breaking down of carbohydrates.<sup>2</sup> These facts raised the probability that glycoll may be synthesized in the animal organism from non-nitrogenous substances chemically related to it.



The experiments<sup>3</sup> recorded below were performed with the object of testing the truth of this theory.

In Table III the results of experiment III are summarized. On the first day the animal received, *per os*, 4.5 grams of sodium benzoate dissolved in warm water. The dose was divided into four equal portions, given about three hours apart. On the second day the animal received 7.5 grams of sodium benzoate and 3

<sup>1</sup>*Zeitschr f physiol Chem*, lxxii, p 491, 1910, *Deutsch med Wochenschr*, xxxvi, p 1432, 1910.

<sup>2</sup>Röhm *Biochemie*, p 540.

<sup>3</sup>Experiments III, IV and V were performed in the laboratories of the Second Medical Clinic of the University of Munich. I wish to express my thanks to Prof. Friedrich von Müller for his kindness in granting me the freedom of his laboratory.

TABLE III  
*Rabbit III*

DATE, 1910	WEIGHT	TOTAL N grams	HIPPURIC ACID grams	HIPPURIC ACID grams	HIPPURIC ACID N TOTAL N	A BENZOIC ACID FLD	B BENZOIC ACID LYE CHLORIDE AS MIN - PUNIC ACID	A B	HIPPURIC ACID PER KG OF BODY WEIGHT	REMARKS
June 9	3 53	2 67	4 42	0 349	13 1	3 81	3 01	79 1	1 25	4 X 1 125 grams of sodium benzoate + 300 cc cream given per os
June 10	3 48	3 47	5 64	0 446	12 8	6 35	3 85	60 6	1 62	5 X 1 5 grams sodium benzoate + 3 grams glycollic acid + 300 cc cream

TABLE IV  
*Rabbit V*

July 19	2 61	0 975	3 56	0 281	17 3	3 39	2 42	71 3	1 37	Animal starving
July 20		1 626								Animal starving 4 grams of sodium benzoate + 2 grams glycollic acid given per os

TABLE V  
*Rabbit IV*

June 29	3 76	1 339				3 39	2 26	66 7	0 91	Starving
June 30	3 63	2 05	3 317	0 262	12 78	3 39	4 12	87 0	1 90	Starving 4 grams of sodium benzoate per os
July 1	3 41	2 59	6 488	0 512	19 8	5 08				Starving 6 grams sodium benzoate

grams of glycollic acid equally distributed into five doses<sup>1</sup> (The glycollic acid had been neutralized by means of sodium bicarbonate) The result was an increase of 12 grams of hippuric acid over the amount in the previous period As is seen from Table V, this increase in the hippuric acid elimination cannot be attributed to the introduction of the glycollic acid, but to the larger dose of benzoic acid The administration of larger doses of benzoic acid, if also accompanied by a simultaneous administration of glycollic acid, was followed by fatal results (Rabbits I and II)

It was observed in experiments I, II and III that not all the benzoic acid administered was eliminated as hippuric acid This phenomenon was explained by previous authors as being due to a lack of glycocoll in the system If this were true, and if glycollic acid actually did go over into glycocoll, then we would expect, on feeding glycollic acid + the benzoate, that the relationship

$$\frac{\text{Benzoic acid fed}}{\text{Benzoic acid excreted as hippuric acid}}$$

would approach 100 per cent But the results in experiment IV (see Table IV) show that this is not the case The  $\frac{A}{B}$  ratio remains as low as in the other experiments

In summing up the results of the last three experiments, we do not find any reasonable ground for the assumption that the glycocoll is synthesized from the non-nitrogenous glycollic acid

On careful consideration, however, of the nitrogen metabolism in the animals during the benzoate period, a possible explanation of the origin of glycocoll suggests itself The goat, during the three days of the foreperiod, excreted an average of 5.6 grams of nitrogen per day Excepting the first day, which may be due to a sudden change in the quantity of food, there is a marked rise in the protein metabolism throughout the course of the benzoate period This was observed in all the experiments and in all the varieties of animals that were experimented upon Furthermore, the increase in the protein destruction, i. e., the increase in the nitrogen elimination above the normal, or above that of a pre-

<sup>1</sup>Rabbits I and II were treated in a similar manner Both died during the course of the second day, owing to the large dose of benzoate Rabbit III died on the day following the experiment Rabbit IV lived for two days after the experiment Rabbit V survived



vious day of a smaller benzoate dose, is always two or three times greater than the amount of nitrogen that has been eliminated as glycocoll in the hippuric acid molecule

Salkowski<sup>1</sup> on giving sodium benzoate, obtained an increase in the nitrogen elimination, accompanied by a corresponding increase in the sulphur metabolism. In experiments VI, VII and VIII (see corresponding tables) we obtained a considerable increase in the total nitrogen on the benzoate days, but the amount of urea remained practically the same as on the previous and following days. Similar results are said to have been obtained by Shepard and Meisner<sup>2</sup>

All these facts suggest the possibility that the glycocoll excreted as hippuric acid did not come from the fraction of protein that would have been metabolized had no benzoate been given, but from the "extra protein" which was destroyed, due to the presence of the benzoic acid. We cannot state with any degree of certainty, what the character of the intermediary process is, but that it is specific and peculiar seems very probable, for none of the extra nitrogen went over into urea. It was all eliminated either as glycocoll or as undetermined nitrogen

#### THE SYNTHESIS OF HIPPURIC ACID

In the dog, the kidney is the only organ in which hippuric acid is synthesized<sup>3</sup>. In the rabbit it can take place even after nephrectomy<sup>4</sup> and is probably brought about by the action of a ferment

Not all of the benzoic acid fed is synthesized into hippuric acid, only an average of 78.1 per cent in the goat (excluding periods VIII and IX for reasons mentioned below) and 73 per cent in the rabbits. It is also to be noted that the relationship between the benzoate fed and the benzoate excreted in the hippuric acid molecule remains fairly constant, except when too large amounts of benzoate are thrown into the system at once, then it sinks, probably because the cells of the body are able to synthesize a certain

<sup>1</sup> Virchow's Archiv, LVIII, p. 530, 1879

<sup>2</sup> Untersuchungen über das Entstehen der Hippursäure, Hanover, 1866 (cited by Wiener, loc. cit.)

<sup>3</sup> Bunge and Schmiedeberg Arch. f. exp. Path. u. Pharm., VI, p. 233, 1877

<sup>4</sup> Salomon Zeitschr. f. physiol. Chem., III, p. 365, 1879

RABBIT VI  
*Rabbit VI*

DATE, 1911	PERIOD	WEIGHT lbs	TOTAL NITROGEN grams	UREA NITROGEN grams	PER CENT OF TOTAL	AMMONIA		PER CENT OF TOTAL	UREA + $\text{H}_2\text{N}$ grams	PER CENT OF TOTAL	HYPERIC ACID N grams	PER CENT OF TOTAL	BEYOZOIC ACID FED grams	REMARKS
						grams	grams							
March 12	I	1 80	0 802	0 7021	87 0	0 0232	2 89	90 49	0 7256	90 49	0 088	7 7	1 7	Starving
March 13	II	1 72	1 147	0 708	69 4	0 036	3 177	72 58	0 829	72 58				Starving
March 14	III	1 60	0 913	0 782	85 67	0 0166	1 82	87 49	0 7986	87 49				Starving

TABUL VII  
Rabbit VII

TABLE VII												
Rabbit VII												
I	1 06	1 192	0 074	51 68	0 080	7 23	1 060	88 91	0 082	0 0	1 7	Starving
II	1 71	1 369	0 907	70 60	0 1127	8 23	1 0797	78 89				Starving
							1 0898	90 9				Starving

March 23

TABLE VIII  
Gord II

Gout II																
	I	II	III	IV	V	1 025	4 255	80 1	0 042	0 85	4 297	87 25	1 210	18 7	12 75	Starving
May 23	10 0					1 025	4 255	80 1	0 042	0 85	4 297	87 25	1 210	18 7	12 75	Starving
May 24						5 154	1 286	93 16	0 053	1 03	4 339	84 19				Starving
May 25						6 501	4 091	72 16	0 156	2 4	4 847	71 50				Starving
May 26						5 001	1 206	85 20	0 086	1 73	4 352	87 02				Starving
May 27						1 478										Starving

amount of benzoate when administered at a slow rate. If it is absorbed at a quicker rate than that at which the cells have the power of combining it with glycocoll, then the benzoate circulates in the blood as such. A good portion of it escapes through the kidney, while another portion is combined with glucuronic acid (compare period VIII of Table I with period VII).

The benzoate, when present in the circulation in large quantities, probably also lowers the "hippuric acid synthesizing function" of the cells. This will explain why in period IX of Table I and in period II of Table III so small an amount of hippuric acid was eliminated (low  $\frac{A}{B}$  ratio).

Magnus-Levy's observation that starving animals eliminate less hippuric acid than do animals which are well nourished is not corroborated by the present findings.

In conclusion, I beg to express my thanks to Prof. Graham Lusk for suggesting this problem and for many valuable suggestions made during the course of the experiment.

#### CONCLUSIONS

1. Goats and rabbits have the power of eliminating hippuric acid containing more glycocoll than is found preformed in the proteins they metabolize. As much as 38.4 per cent of the total nitrogen of the goat was eliminated as glycocoll nitrogen in the hippuric acid.

2. The ingestion of benzoate in large quantities results in a considerable increase in the nitrogen elimination. The production of urea is not affected by it.

3. The increase in the nitrogen elimination is much greater than the amount of nitrogen eliminated in the hippuric acid.

4. It is suggested that the large quantities of glycocoll originate from the "extra destroyed" protein, and not from the protein that would have been metabolized had no benzoate been given.

5. The diet has no influence on the amount of hippuric acid eliminated.

6. No synthetic production of glycocoll from glycollic acid could be determined.

7. A suckling calf, fifteen days old, which had never received the glycocoll complex in its food, was in full possession of the power to synthesize hippuric acid and eliminate it in large quantities.

## STUDIES IN NUTRITION

### II THE UTILIZATION OF THE PROTEINS OF BARLEY

BY LAFAYETTE B MENDEL AND MORRIS S FINE

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(Received for publication, September 25, 1911)

Data regarding the utilization of the proteins of barley are confined almost entirely to the Japanese literature,<sup>1</sup> according to which they are but 40 to 76 per cent available. However, the experiments from which these data are taken were not exempt from those unfavorable conditions which render it difficult to draw conclusions.

#### METHODS

The routine incident to the metabolism experiments did not differ essentially from that described in the previous paper of this series. The concentration of hemicelluloses in the barley preparation and in the resulting feces was determined. For this purpose 2.5 to 3.5 grams of the dried material were boiled for four hours with 220 cc of 2 per cent hydrochloric acid.<sup>2</sup> Proteoses and peptones, which disturb the subsequent precipitation of copper oxide, were removed by the addition of a solution of phosphotung-

<sup>1</sup> Cf. Oshima U. S. Department of Agriculture, Office of Experiment Stations, Bull. 159, 1905.

<sup>2</sup> Cf. Mendel and Fine. *This Journal*, v, p. 303, 1911.

<sup>3</sup> From the studies of Swartz (*Transactions of the Connecticut Academy of Arts and Sciences*, xvi, p. 323, 1911) it appears that maximum reduction after hydrolysis is obtained in three to four hours. The crude barley protein preparation of these experiments yielded but 0.6 per cent more hemicelluloses when boiled four hours than when the interval was reduced to two and a half hours as called for in the official method for the estimation of starch (U. S. Dept. of Agriculture, Bureau of Chemistry, Bull. 107, p. 53, 1910).

stic acid<sup>1</sup> The resulting precipitate was washed, the united filtrates neutralized and made up to 500 cc, of which 25 cc were used for the sugar estimation according to the Allihn gravimetric method The hemicellulose was computed by multiplying the dextrose thus obtained by 0.9<sup>2</sup>

## EXPERIMENTAL PART

### *Product Employed*

The crude barley protein employed in this study was prepared as follows

About seven pounds of barley flour,<sup>3</sup> in one pound lots, were made into a thin homogeneous mush with water and heated in an autoclave at about 120°C for ten to twenty minutes After the material had cooled to a temperature of about 60°C an amylolytic preparation was added It was necessary to repeat this process at least three times before the cells were completely disintegrated and no starch reaction was obtained in a test tube trial The insoluble material thus obtained settled very readily and was washed with ease four to six times with water by decantation It was finally filtered, dried on a water bath and ground to an impalpable powder Analysis gave the following values

	per cent
Protein ( $N \times 5.74$ )	51.0
Carbohydrate by hydrolysis (hemicelluloses)	21.7
Ether extract	8.3
Ash	1.0
Moisture	3.0
Crude fiber (by difference)	15.0

When treated with iodine and examined under the microscope, starch particles were so infrequent as to be considered absent, yet

<sup>1</sup> Cf. *Abderhalden's Handbuch der Biochemischen Arbeitsmethoden*, III, 1, p. 271, 1910 With this method the barley preparation yielded 21.7 per cent hemicelluloses, when the phosphotungstic precipitation was omitted, the yield was reduced to 18.5 per cent Swartz (*loc cit*, p. 343) adopted the expedient of clarifying with charcoal with satisfactory results

<sup>2</sup> This is believed to be justifiable in view of the similarity in ultimate analysis of starch and hemicelluloses

<sup>3</sup> Mr. M. F. Deming of the Cereo Company, Tappan, N. Y., kindly contributed this material

<sup>4</sup> Factor proposed by Atwater and Bryant *Report of the Storrs Agricultural Experiment Station*, p. 79, 1899

on hydrolyzing with dilute acid, 22 per cent of carbohydrate was obtained. This condition would suggest that the values for starch, as ordinarily found by acid hydrolysis, cannot be relied upon, since a body other than starch may be present, which on hydrolysis yields reducing substances. Indeed Schulze<sup>1</sup> has shown the wide-spread occurrence of hemicelluloses, a group of substances readily attacked by dilute acids, yielding reducing sugars, and only very slowly affected by enzymes.<sup>2</sup> Apparently the untreated barley contains at least 5 per cent of hemicelluloses.

### *Metabolism Experiments*

Crude barley protein was fed to two bitches as detailed in the first two tables. Casual inspection of these data would indicate that barley protein is relatively poorly utilized, having a coefficient of digestibility of 85 per cent against 97 per cent for meat fed, as it might at first sight appear, under identical conditions. However, each day's supply of barley protein preparation contained about 8 grams of crude fiber—practically indigestible<sup>3</sup>—and 11 grams of hemicelluloses, of which 9 grams reappeared in the feces in both experiments.<sup>4</sup> The barley-protein feces thus contained about 17 grams of undigested non-nitrogenous material, and these experiments should therefore not be held comparable to trials with meat where such unfavorable conditions were not in evidence.

<sup>1</sup> Cf. Schulze and Godet, *Zeitschr. f. physiol. Chem.*, lxi, p. 281, 1909.

Cf. Swartz, *Loc. cit.* (contains the literature).

<sup>2</sup> Cf. Swartz, *Loc. cit.*, p. 268 ff. (contains the literature).

<sup>4</sup> The barley-protein feces from both animals yielded 10 grams of dextrose (= 9 grams of hemicellulose) on hydrolysis. The criticism might be offered that a portion of the dextrose thus obtained is due to the cane sugar of the food mixture, its digestion and absorption having been diminished by the excessive fecal discharges (22 grams dry daily). Against this objection are the following facts: (1) meat diets containing 10 grams of bone ash but otherwise identical to those in this paper yielded 14 to 15 grams of dry carbohydrate-free feces daily, (2) cotton seed diets, containing 25 grams of cane sugar as in the above trials, produced 23 to 24 grams of dry feces, which yielded only 4 to 6 grams of reducing carbohydrate on hydrolysis—quantities attributable to undigested hemicelluloses of the food.

The importance of comparing experiments in which the indigestible non-nitrogenous material,<sup>1</sup> *in addition to* the nitrogen intake and accessory articles of diet, are similar, was adequately appreciated only after the major portion of the studies of this series was completed. Trials in which these principles were consistently applied not being at hand, we must be content with grouping data which will as far as possible enable one to interpret properly the results on protein utilization. Table 3 contains such an arrangement. Meat diets containing 6 grams of fiber and 13 grams of indigestible materials were 91 and 89 per cent utilized, respectively. Thus the utilization of 85 per cent for the barley preparation with its 8 grams of fiber and 11 grams of hemicelluloses (of which 9 grams reappeared in the feces) leads one to believe that *under favorable conditions, barley protein*, like that of the closely related cereal wheat, *would be almost perfectly digested*.

TABLE 1  
*Crude Barley Protein*

SUBJECT DOG 5 Weight at beginning 62 kg and 63 Kg		Weight at	PERIOD XXIII (5 days) Meat Feeding	PERIOD XXIV* (4 days) Barley Protein Feeding
Composition of daily diet			grams	grams
			Meat 150	Barley protein 52
			Sugar 25	Sugar 25
			Lard 20	Lard 30
			Water 100	Water 200
			Estimated calories 530	Estimated calories 490
			Daily Averages	Daily Averages
<i>Nitrogen output</i>				
Urine nitrogen, gm			3 68	3 99
Total nitrogen, gm			3 84	4 67
Nitrogen in food, gm			4 64	4 64
Nitrogen balance, gm			+0 80	-0 03
<i>Feces</i>				
Weight air dry, gm			3 4	22 0
Nitrogen, gm			0 16	0 68
Nitrogen, per cent			4 62	3 10
Nitrogen utilization, per cent			96 6	85 3

\*About half the food forced

<sup>1</sup> The influence of such substances upon nitrogen utilization will be discussed more fully in a subsequent paper

TABLE 2  
*Crude Barley Protein*

SUBJECT DOG 7 Weight at beginning 66 Kg    Weight at end 67 Kg		PERIOD XXVIII (5 days) Meat Feeding	PERIOD XXIX (5 days) Barley Protein Feeding
Composition of daily diet		grams	grams
		Meat 150	Barley protein 52
		Sugar 25	Sugar 25
		Lard 20	Lard 30
		Water 100	Water 200
		Estimated calories 530	Estimated calories 490
<i>Nitrogen output</i>		Daily Averages	Daily Averages
Urine nitrogen, gm		3 60	3 75
Total nitrogen, gm		3 75	4 45
Nitrogen in food, gm		4 64	4 64
Nitrogen balance, gm		+0 89	+0 19
<i>Feces</i>			
Weight, air dry, gm		3 8	22 4
Nitrogen, gm		0 15	0 70
Nitrogen, per cent		3 82	3 12
Nitrogen utilization, per cent		96 9	85 0

TABLE 3

*Utilization with Reference to Indigestible Materials in the Diet Daily Averages*

DOG	PERIOD	DAYS	NATURE OF INGESTA	FIBER IN OR ADDED TO FOOD	TOTAL INDIGES- TIBLE MATERIAL IN FOOD	N INTAKE	N UTILIZA- TION	AVER- AGE N UTILIZA- TION
				grams	grams	grams	per cent	per cent
5	XXIX	4	Crude barley protein	8	19†	4 6	85 3	85 2
7	XXIX	5		8	19†	4 6	85 0	
5	VIII*	4	Meat	6	6	3 3	90 5	91 0
6	XIX*	4		6	6	3 3	89 2	
7	VIII*	4		6	6	3 3	93 3	
5	VI*	4	Meat	6	13	3 3	91 6	89 2
6	VI*	4	Agar‡ 2 gm	6	13	3 3	87 7	
7	VI*	4	Bone ash§ 5 gm	6	13	3 3	88 3	

\* The details of these experiments will be published in a subsequent paper of this series

† This includes 11 gram of hemicelluloses which were 18 per cent utilized (see page 341 this paper)

‡ Salki (this Journal 11 p 251 1906) recovered all but 17 per cent of the agar fed. The amount of agar thus failing to reappear in the feces of these experiments is too small to be considered

§ We are unable to state exactly how completely the bone ash reappears in the feces. Steel and Gies (Amer Journ of Physiol xx, p 350 1907) found no change in urinary calcium or phosphorus when large amounts of bone ash were added to the diet





## STUDIES IN NUTRITION

### III THE UTILIZATION OF THE PROTEINS OF CORN

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Corn has been produced extensively as a food for man in America for many hundreds of years<sup>1</sup> It is scarcely a century since corn has ceased to be the most important of our food cereals, this high place in our dietary having since been assumed by wheat because of its superior bread-making qualities Corn has been, and is, an important article of diet in other parts of the world also A very popular dish in Italy is maize cooked with water to a stiff mush, to which a little cheese is added This preparation is called *polenta*, and according to Ranke<sup>2</sup> it is the main article of diet in certain parts of Italy

Rubner reported a study on the availability of *polenta* seasoned with meat extracts The digestibility of the whole was 85 per cent, but assuming the meat extracts to be completely absorbed, the apparent utilization would fall to 80 per cent

In experiments on himself Malfatti found the digestibility of maize to be 82 per cent The utilization of this material was lowered to 68 per cent by the addition of much butter and raised to 93 per cent when cheese was added to the diet

The work of Grandeau, on the availability of maize in the horse, is of interest in that it shows that even in an animal with a long intestine, where the food may remain for a greater length of time, and the cellulose be dissolved to a considerable extent—even here the digestibility was an average of only 69 per cent The generally low utilization of *polenta* is further shown by Albertoni and Novi, and by Erismann

<sup>1</sup> Cf Merrill (1906) see bibliography

<sup>2</sup> Ranke *Zeitschrift für Biologie*, VIII, p 130, 1877

The utilization experiments of Merrill, in which the protein of the corn of various corn preparations was found to vary in digestibility from 73 to 86 per cent, lend further support to these data, as does the later work of the same author, in which the protein of corn was calculated to be only 61 per cent utilizable.

The above data on the digestibility of maize are quite contrary to those obtained for "roborat,"<sup>1</sup> an albumose-like preparation. "Roborat" has been the subject of considerable investigation, notably by Laves, Loewy and Pickart, Wintgen, Hoppe, and Sommerfeld, the consensus of opinion being that this commercial material is quite as well utilized as meat.

Pertinent objections may be raised to all the foregoing data on the digestibility of the proteins of corn. The maize employed contained considerable starch, and it is an open question as to what extent the cells were ruptured. The unfavorable influence of these conditions has been discussed at length in a previous paper.<sup>2</sup> While these objections do not apply to the thoroughly digested "roborat," still here the protein has been considerably changed, being present in part as proteoses.

Investigations on the pure unchanged protein would of course be free from these criticisms. Maize contains several proteins,<sup>3</sup> one of which is zein, an alcohol-soluble protein, which makes up about half the total protein. The experiments on zein are practically limited to those of Rockwood on dogs. In two experiments this observer found the utilization of zein to be 78 and 90 per cent respectively. It should be noted that the zein employed by Rockwood was hard and not very finely divided, and it is possible that this may have been a factor contributing to the poor utilization.

Henriques has studied zein with reference to its ability to maintain nitrogenous equilibrium in rats. Incidentally one notes from his tables that the zein varied in its apparent utilization between 50 and 90 per cent. However, zein, which had been previously subjected to tryptic digestion was only 74 per cent utilized,

<sup>1</sup> For an account of the properties of this material compare Loewy and Pickart (see bibliography).

<sup>2</sup> Cf. Mendel and Fine. *This Journal*, v, p. 303, 1911.

<sup>3</sup> See T. B. Osborne. "Die Pflanzenproteine," *Ergebnisse der Physiologie*, v, p. 47, 1910.

and zein obtained by precipitation from its solution in alkali with acid was but 64 per cent digested Szumowski's<sup>1</sup> observations may be of interest in this connection A 4 kg dog was fed with a mixture of 100 grams of finely ground zein, sugar, lard and water Five hours later the dog was bled to death, and there were recovered 61 grams of zein from the stomach, 18 grams from the small intestine and 6 grams from the large intestine In another instance, a solution of alkali-zein was fed, which resulted in irritation of the stomach and diarrhoea It may be that the poor utilization obtained by Henriques for zein subjected to tryptic digestion, and for the zein previously dissolved in alkali, is due to the production of diarrhoea Indeed it is well known that proteose preparations from meat and other sources may show apparently poor utilization for this very reason

The literature thus assembled would seem to favor the view that the unchanged protein of corn is poorly utilized, but it should be borne in mind that the conditions attending these experiments have in practically no instance been free from objection

## EXPERIMENTAL PART

### *Product Employed*

The present studies were confined to corn gluten,<sup>2</sup> which contained practically no starch A large amount of this substance was obtained by Dr T B Osborne, who very kindly supplied all of this material necessary for our experiments

### *Metabolism Experiments*

The gluten, ground to an impalpable powder, was fed to three bitches, the usual method of procedure prevailing<sup>3</sup> Dogs 6 and 7 (Tables 2 and 3) in the experimental periods received no other nitrogenous substance except the corn gluten, while dog 5 (Table 1) received two-thirds of the total nitrogen as corn gluten, and the remainder in the form of meat

<sup>1</sup> Szumowski *Zeitschrift für physiologische Chemie*, xxxvi, p 198, 1902

This material contained 7.6 per cent nitrogen

<sup>2</sup> See Mendel and Fine *This Journal*, x p 303, 1911

TABLE 1  
Corn Gluten with Agar and Bone Ash

SUBJECT, DOG 5 Weight at beginning, 5 7 Kg Weight at end, 5 4 Kg	PERIOD I (5 days) Meat Feeding	PERIOD II (4 days) Corn Gluten and Meat Feeding	PERIOD III (4 days) Meat Feeding
Composition of daily diet	grams	grams	grams
	Meat 160	Corn 150	Meat 150
	Sugar 20	gluten 43	Sugar 20
	Lard 20	Meat 50	Lard 20
	Agar 3	Sugar 20	Agar 3
	Bone ash 7	Lard 25	Bone ash 7
	Water 100	Agar 3	Water 100
		Bone ash 7	
		Water 175	
	Estimated calories 530	Estimated calories 470	Estimated calories 520
		Corn gluten furnished 66 6 per cent of the total nitrogen	
	Daily Averages	Daily Averages	Daily Averages
Nitrogen output			
Urine nitrogen, gm	4 44	4 21	3 80
Total nitrogen, gm	4 73	4 72	4 15
Nitrogen in food, gm	4 89	4 91	4 90
Nitrogen balance, gm	+0 16	+0 19	+0 75
Feces			
Weight air dry, gm	13 2	23 5	14 5
Nitrogen, gm	0 29	0 51	0 36
Nitrogen, per cent	2 22	2 16	2 48
Nitrogen utilization, per cent	94 0	89 7	92 7

TABLE 2  
Corn Gluten with Agar and Bone Ash

SUBJECT, DOG 6 Weight at beginning, 5 3 Kg Weight at end, 5 1 Kg	PERIOD I (4 days) Meat Feeding	PERIOD II (5 days) Corn Gluten Feeding	PERIOD III (5 days) Corn Gluten Feeding	PERIOD IV (5 days) Meat Feeding
Composition of daily diet	grams	grams	grams	grams
	Meat 150	Corn 150	Corn 150	Meat 150
	Sugar 20	gluten 65	gluten 65	Sugar 20
	Lard 20	Sugar 20	Sugar 20	Lard 20
	Agar 3	Lard 25	Lard 25	Agar 3
	Bone ash 7	Agar 3	Agar 3	Bone ash 7
	Water 100	Bone ash 7	Bone ash 7	Water 100
		Water 175	Water 200	
	Estimated calories 520	Estimated calories 420	Estimated calories 420	Estimated calories 520
	Daily Averages	Daily Averages	Daily Averages	Daily Averages
Nitrogen output				
Urine nitrogen, gm	3 89	4 79	4 71	3 70
Total nitrogen, gm	4 16	5 29	5 17	4 06
Nitrogen in food, gm	4 93	4 95	4 95	4 93
Nitrogen balance, gm	+0 77	-0 34	-0 22	+0 87
Feces				
Weight, air dry, gm	12 2	26 0	21 0	13 6
Nitrogen, gm	0 28	0 50	0 46	0 36
Nitrogen, per cent	2 28	1 92	2 17	2 64
Nitrogen utilization, per cent	94 3	89 9	90 7*	92 7

\*By an accident, about half the feces of this period were charred giving rise to possible inaccuracies

TABLE 3  
*Corn Gluten with Agar and Bone Ash*

SUBJECT, DOG 7 Weight at beginning 5.6 Kg Weight at end 4.9 Kg	PERIOD I (4 days) Meat Feeding	PERIOD II (4 days) Corn Gluten Feeding	PERIOD III (5 days) Meat Feeding
	grams	grams	grams
Composition of daily diet	Meat 100	Corn 100	Meat 100
	Sugar 20	gluten 43	Sugar 20
	Lard 20	Sugar 20	Lard 25
	Agar 3	Lard 25	Agar 3
	Bone ash 7	Agar 3	Bone ash 7
	Water 100	Bone ash 7	Water 100
		Water 160	
	Estimated calories 430	Estimated calories 390	Estimated calories 470
	Daily Averages	Daily Averages	Daily Averages
Nitrogen output			
Urine nitrogen, gm	2.98	3.30	2.58
Total nitrogen, gm	3.26	3.66	2.81
Nitrogen in food, gm	3.29	3.27	3.29
Nitrogen balance, gm	+0.03	-0.39	+0.48
Feces			
Weight air dry, gm	12.5	19.2	12.8
Nitrogen, gm	0.28	0.36	0.23
Nitrogen, per cent	2.26	1.86	1.81
Nitrogen, utilization, per cent	91.4	89.0*	93.0

\* By an accident about half the feces of this period were charred giving rise to possible errors in analysis

From the accompanying brief summary it is apparent that the protein of the corn gluten is only slightly less well utilized than meat similarly fed

*Summary of the Data on Nitrogen Utilization (see Tables 1-3)*

DOG	CORN GLUTEN	MEAT (AVERAGE)
	per cent	per cent
5	89.7	93.3
6	89.9	93.5
6	90.7	93.5
7	89.0	92.2

Certain considerations lead us to believe that under ideal conditions the utilization of corn would compare *even more favorably* with that of meat. The proportion of protein to crude fiber in corn flour<sup>1</sup> is approximately 8:1. It is probable therefore that the corn gluten of our experiments had a crude fiber concentration approximating 6 per cent. In the light of experiments to be reported in detail in a subsequent paper, it is conceivable that the cellulose, etc., thus included in the diet would in part account for the difference in the coefficients of digestibility of the proteins of corn and meat.

TABLE 4

*Utilization with Reference to Indigestible Materials in the Diet Daily Averages*

DOG	PERIOD	DAYS	NATURE OF INGESTA	FIBER IN OR ADDED TO FOOD	TOTAL INDIGESTIBLE MATERIAL IN FOOD	N INTAKE	N UTILIZATION	AVERAGE N UTILIZATION
			grams	grams	grams	grams	per cent	per cent
5	VI*	4	Meat	6	13	3.3	91.6	89.2
6	VI*	4	Bone ash	6	13	3.3	87.7	
7	VI*	4	Agar	6	13	3.3	88.3	
6	II	5	Corn gluten	4	14†	4.9	89.9	89.9
6	III	5	Bone ash	4	14†	4.9	90.7	
7	II	4	Agar	3	13†	3.3	89.0	

\* The details of these experiments appear in a subsequent paper in this series.

† These values are low if any thing, a certain amount of indigestible hemicelluloses probably being present.

From Table 4 it is apparent that *meat diets* containing amounts of indigestible materials comparable to those in the corn gluten diets were no more thoroughly utilized than the corn gluten. *From this point of view, the proteins of corn are evidently quite thoroughly utilized.*

There may be some doubt as to the validity of certain of the data in Tables 2 and 3 as is pointed out in foot-notes to the tables, but the data are in such close accord with others, about which there are no such uncertainties, that we are inclined to accept them as giving true pictures of the actual state of affairs.

<sup>1</sup> See Atwater and Bryant U. S. Department of Agriculture, Office of Experiment Stations, Bull. 28 (revised), p. 56, 1906.

A rather unsuccessful attempt was made to determine directly the influence of indigestible materials upon the utilization of corn proteins. Agar and bone ash were omitted from the food mixtures (See Table 5). A new sample of gluten was employed, toward which the animals acted very differently than they did toward the first sample. Dogs 5 and 6 vomited practically all their food, and dog 7 yielded results which cannot be accepted entirely without question, since, as pointed out in the foot-note to the table, a portion of the food here also was ejected. Here the utilization of the corn protein is decidedly inferior to that of meat, and even thus, the apparent utilization recorded is better than is actually the case, since not all the food noted in the table was submitted to digestion. Why the animals acted so differently toward these two samples of corn gluten, we are unable to explain. Although quite finely ground, the second sample was not an impalpable powder, and this may account for its low digestibility. This explanation is not especially satisfactory to us.

TABLE 5

*Corn Gluten without Agar and Bone Ash*

SUBJECT DOG 7 Weight at beginning 6.6 kg Weight at end 6.6 kg		PERIOD XXVII* (3 days) Corn Gluten Feeding	PERIOD XXVIII (5 days) Meat Feeding
		grams	grams
Composition of daily diet	Corn gluten	61	Meat 150
	Sugar	25	Sugar 25
	Lard	25	Lard 20
	Water	225	Water 100
	Estimated calories	450	Estimated calories 530
		Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm		4.38	3.60
Total nitrogen, gm		4.78	3.75
Nitrogen in food, gm		4.76	4.64
Nitrogen balance, gm		-0.02	+0.89
<i>Feces</i>			
Weight air dry, gm		10.7	3.8
Nitrogen, gm		0.41	0.15
Nitrogen per cent		3.82	3.82
Nitrogen utilization, per cent		91.4	96.9

For the first two days the food was greedily eaten; on the last day a small part of the food had to be forced. About one-tenth of the food of the last day was vomited.



From Table 6 it is apparent that with one exception *negative balance resulted in the corn gluten periods while positive balances obtained throughout the meat periods*. The one exception is that of Dog 5, and in this case it will be recalled that one-third of the daily nitrogen was supplied by meat. Henriques failed to establish nitrogenous equilibrium with zein in rats.

TABLE 6  
*Average Daily Balances — Nitrogen, in grams*

SUBJECT	TABLE	GLUTEN PERIODS	CORRESPONDING MEAT PERIODS
Dog 5	1	+0 19	+0 16, +0 75
Dog 6	2	-0 34, -0 22	+0 77, +0 87
Dog 7	3	-0 39	+0 03, +0 48
Dog 7	5	-0 02	+0 59

#### SUMMARY

Corn proteins, partially purified, were somewhat less thoroughly utilized than meat. Evidence was presented to indicate that this small difference may in great part be attributed to the cell residues remaining in the corn preparation employed.

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# STUDIES ON THE FORMATION OF GLYCOCOLL IN THE BODY I<sup>1</sup>

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(Received for publication, October 12, 1911)

Considerable interest has been added to the importance of the study of the metabolism of the amino-acids by the more recent work on sugar production from these substances in diabetes. In a paper by Ringer and Lusk<sup>2</sup> experimental evidence is at hand of the conversion of glycocoll, l-alanine and glutamic acid into sugar.

A knowledge of the total extent to which these amino-acids can be formed in the body would afford us a better understanding of the processes involved in the intermediate metabolism of proteins. The ease with which benzoic acid can evidently cause the production of glycocoll, and consequently the elimination of hippuric acid, affords us a simple means of examining more deeply into at least one phase of the cleavage of proteins in the body.

The subject of hippuric acid has received considerable attention from a number of investigators, and much valuable information concerning it is recorded. It is known from the work of Parker and Lusk,<sup>3</sup> Wiechowski,<sup>4</sup> Magnus-Levy,<sup>5</sup> and others, that glycocoll can be eliminated as hippuric acid to a very unusual extent, on feeding benzoic acid. It has been ascertained that glycocoll so eliminated can constitute the major part (up to 64 per cent) of the total nitrogen present in the urine.

The results of nearly all investigators agree in showing that the glycocoll formation does not depend upon the amount of protein

<sup>1</sup> Aided by the George Blumenthal Jr. fellowship.

<sup>2</sup> Ringer and Lusk. *Zeitschr. f. physiol. Chem.*, lxxvi, Heft 1 and 2.

<sup>3</sup> Parker and Lusk. *Amer. Journ. of Physiol.*, iii, No. 9.

<sup>4</sup> Wiechowski. *Hofmeister's Beiträge*, vii, 1906.

<sup>5</sup> Magnus-Levy. *Münchener med. Woch.*, No. 52, p. 2168, 1905.

metabolism, but to a certain extent goes hand in hand with the amount of benzoic acid administered within a given period. *In vivo* no body-protein can yield on hydrolysis much over 3 per cent of glycocoll. However, the amount of glycocoll which the body can furnish for synthesis into hippuric acid is far in excess of that performed in such proteins. The origin of this excess of glycocoll remains unexplained. It has been assumed by some investigators that the animal body contains a "surplus" or reserve of glycocoll (presumably like that of glycogen) which is drawn upon by the benzoic acid. Such an assumption necessarily implies that under certain conditions the supply of glycocoll (similar to that of glycogen) can be exhausted. Magnus-Levy<sup>1</sup> questions this hypothesis and arrives at a different conclusion. Judging by the results obtained by most investigators the idea of a reserve or surplus does not seem to hold.

As stated above it is generally admitted that within certain limits the production and elimination of glycocoll run parallel with the amount of benzoic acid fed. This coercive action of benzoic acid upon the formation and elimination of glycocoll reaches a maximum—which maximum, however, as Wiechowski has shown, can be maintained after a prolonged period, a similar observation was made in work on dogs by Salkowski.<sup>2</sup> In our own series of experiments, one rabbit proved unusually resistant to benzoic acid and the drug was fed over a prolonged period. This rabbit (R 5), weighing 1700 grams, received daily 1.7 grams of benzoic acid (1 gram per kilo) in the form of sodium benzoate, subcutaneously for fifteen days with the following result:

I Normal period—four days (average of daily determinations) 0.556 gram total N, 0.012 gram hippuric acid N

II First benzoic period—fifteen days (average of daily determinations) 0.882 gram total N, 0.131 gram hippuric acid N

Computing for the entire period of fifteen days, the animal eliminated 13.230 grams of nitrogen of which 1.965 grams or 14.8 per cent was hippuric acid nitrogen.

III Without allowing any normal interval, the animal was given daily 2.532 grams benzoic acid (1.5 grams per kilo) for eleven consecutive days, and

<sup>1</sup> Magnus-Levy *loc. cit.*

<sup>2</sup> Salkowski *Internat. Beitr. zur Medizin*, 11, p. 27, 1902.

it eliminated in this period 0.916 gram total N, and 0.168 gram hippuric acid N as a daily average

The total nitrogen for eleven days amounted to 10.076 grams of which 1.848 grams or 18.3 per cent was hippuric acid N. In short, the animal received benzoic acid daily for twenty-six successive days with the result that it catabolized 145.7 grams of protein (23.306 grams N) yielding at the same time 20.5 grams of glycocoll (3.813 grams N) as hippuric acid.

Computing from the normal period given above, the probable metabolism of twenty-six normal days would amount to 90.5 grams protein with a glycocoll yield of 1.6 grams. The extra glycocoll yield obtained in the benzoic acid period, however, was 18.9 grams from 55 grams of extra proteid catabolized, that is to say, 34 per cent.

Several points of interest appear in this experiment. It is seen first of all, that though for a given amount of benzoic acid (1 gram per kilo), the animal does not (on an average) synthesize daily more than 70 per cent of it into hippuric acid, the glycocoll is evidently inexhaustible when the entire period is considered. It appears, moreover, that an additional 0.5 gram of benzoic acid per kilo causes a rise in both the total nitrogen and hippuric acid nitrogen output. In this period, however, the total benzoic acid synthesized daily is only 58 per cent. In other words, notwithstanding the total rise in the hippuric acid output, the relative coercive efficiency of the benzoic acid for the production of glycocoll drops. This may be interpreted as an indication of the fact that no real reserve or surplus of glycocoll exists in the body, but that it is formed progressively as the need for it arises.

In Wiechowski's work<sup>1</sup> and our own, we find indications that the hippuric acid elimination depends upon four factors, namely, (1) the amount of benzoic acid present in the circulation at a given time, (2) the extent of glycocoll formation, (3) the rate of glycocoll formation in the body, and (4) the rate of hippuric acid synthesis. The first of these—the amount of benzoic acid in the circulation—naturally depends upon the amount of benzoic acid given, and consequently the rate of elimination of benzoic acid as such by the kidneys and intestines. As the above experiments (and all other experiments of this character) show, the extent of glycocoll formation is partly dependent upon the amount of benzoic acid given. This is well demonstrated in the increase of glycocoll output on increasing the amount of

<sup>1</sup> Wiechowski *loc. cit.*

benzoic acid on the sixteenth day of the experiment. The fall in the efficiency of the benzoic acid to synthesize glycocoll may be due partly to the inadequate rate of glycocoll formation and partly to the rate and extent of free benzoic acid elimination.

The excess of the daily total nitrogen output in the first benzoic period (II) of the above experiment, over that of the normal period (I) (i.e., extra nitrogen) is 0.326 gram, of which 0.119 gram or 37 per cent is hippuric acid nitrogen. The daily total nitrogen of the second benzoic period (III) exceeds that of the first (II) by 0.034 gram and the extra glycocoll nitrogen which appears in this period is 0.037 gram, but the extra nitrogen eliminated in consequence of an additional 0.5 gram (per kilo) of benzoic acid, appears entirely in the form of hippuric nitrogen. This is important and we shall revert to it later.

If we analyze the daily results of this experiment (see Table 2) and of many others in our series (excepting those made on fasting animals), we find that the largest total nitrogen and hippuric acid output occurs on the first day of each period. On the subsequent days of the continued period both total nitrogen and hippuric acid are less in amount, reaching a level for a constant dose of benzoic acid which is indefinitely maintained. The fall affects principally the total nitrogen. The hippuric acid nitrogen output diminishes but slightly. It is only at the beginning of each period, therefore, that the extra nitrogen eliminated on administering benzoic acid is three to four times as much as that present in the form of hippuric acid. Later on the two values tend to approach each other.

Wiechowski maintains, from similar observations, that the level of hippuric acid production which is reached after a certain time can not be changed. This, however, is not wholly the result of the animal's synthetic capacity of glycocoll and benzoic acid (as Wiechowski would lead us to believe) nor of the lack of glycocoll in the body, but it is evidently due to another factor, namely to the coercive efficiency of the benzoic acid itself to cause the production of glycocoll, as shown in our prolonged benzoic acid experiment, when on the sixteenth day the dose of benzoic acid is suddenly increased. It is true that the increase in the hippuric acid nitrogen output at that time is not very great, nor is it proportionate to the increase in the dosage of benzoic acid, and yet the extra hippuric acid nitrogen eliminated amounts to about 25 per

TABLE 1

TREATMENT	DATE	WEIGHT OF ANIMAL	FOOD		URINE		
			Ons	Carrots	Total N	Hippuric acid N	Difference N
		grams	grams	grams	grams	grams	grams
<i>Rabbit 6</i>	January 13	2110	10	70	0 854	0 025	0 829
	January 14	2120	40	130	0 840	0 016	0 824
	January 15	2130	40	150	1 085	0 218	0 867
	January 16	2150	10	95	1 071	0 162	0 909
	January 17	2150	40	83	0 987	0 117	0 870
<i>Rabbit 7</i>	January 18	2120	40	40	0 875	0 121	0 754
	February 6	2260	40	150	1 267	0 028	1 239
	February 7	2250	40	130	1 155	0 016	1 139
	February 8	2210	40	105	0 917	0 032	0 885
	February 9	2200	0	90	1 859	0 151	1 708
<i>Rabbit 8</i>	February 10	2170	0	90	1 575	0 133	1 442
	February 11	2130	40	150	1 362	0 125	1 237
	February 12	2200	40	100	1 042	0 124	0 918
	February 13	2110	00	150	1 036	0 016	1 020
	February 14	2150	30	200	1 007	0 016	0 991
<i>Rabbit 9</i>	February 15	2170	110	110	1 573	0 193	1 380
	February 16	2100	170	170	1 191	0 181	1 010
	February 17	2100	150	150	1 365	0 107	1 258
	February 18	2100	150	150	1 365	0 107	1 258
	February 19	2100	150	150	1 365	0 107	1 258

TABLE 2

PERIOD	DATE	WEIGHT OF ANIMAL	FOOD		URINE		
			Oats	Carrots	Total N	Hippuric Acid N	Extra N
Rabbit 5		grams	grams	grams	grams	gram	gram
	I Normal period				average of 4 days		
					0 644	0 012	
					0 539		
					0 518		
					0 525		
					1 107		
					0 994		
					0 938		
					0 833		
II Benzonic acid 10 gram per kilo					0 805		0 551
					0 987		0 438
					0 847		0 382
					0 875		0 277
							0 279
III Benzonic acid 15 grams per kilo							0 431
							0 291
							0 319

\* This effect of change in dosage upon the total nitrogen and hippuric acid nitrogen elimination is more marked when the benzoic acid is administered hypodermically than when given *per os*

cent of the total hippuric acid nitrogen excreted on the previous days. We would therefore be inclined to lay some stress upon the capacity of benzoic acid for the production of glycocoll as well as upon its power to induce the synthesis of hippuric acid. A sudden rise in the total nitrogen and hippuric acid nitrogen, with a subsequent fall to a constant level, is observable not only after every normal period or interval—using a like amount of benzoic acid—but also during continued periods of benzoic acid feeding, when the dose is suddenly increased.

It is the extra nitrogen, other than that of glycocoll origin therefore, which is the more subject to variation. Unfortunately there is no record at hand of the exact nature of the extra nitrogen, which appears in the urine at the inception of each period, that is not glycocoll. Ringer<sup>1</sup> records the results of experiments on two rabbits "which show that the amount of urea nitrogen plus ammonia nitrogen during the benzoic period does not differ from normal days."

We have but one series of experiments on the rabbit in which the urea nitrogen and ammonia nitrogen were determined and in this series also (as shown in the following table) neither the urea nor the ammonia rise above that in the normal period.

TABLE 3

DATE	TOTAL N	THREE DAYS TOTAL N	UREA N	NH <sub>3</sub> N	HIP- PURIC ACID N	
<i>Rabbit 1</i>	<i>gram</i>	<i>grams</i>	<i>grams</i>	<i>gram</i>	<i>gram</i>	
September 18	0 602	1 624	1 396	0 027		Benzoic acid given, 1 020 grams or 0 5 gram per kilo
September 19	0 567					
September 20	0 455					
September 21	0 727	2 078	1 386	0 101	0 112	
September 22	0 721					
September 23	0 630					
September 24	0 637	1 806	1 418	0 033	0 019	
September 25	0 595					
September 26	0 574					

<sup>1</sup> Ringer. On the Origin of Glycocoll in the Animal Body, *Proc Soc for Exp Biology and Medicine*, viii, No 4, April 19, 1911.



It would be hardly justifiable to draw conclusions from our set of experiments, the results are in accord with those obtained by Ringer, showing that urea N plus ammonia N remain unaltered.

The fluctuation in the extra nitrogen is evidently not due to extraneous influences, such as the amount or character of food consumed. In experiments on fasting animals the glycocoll output as hippuric acid follows the same general rule after benzoic acid administration, as previously described, the total nitrogen (see Table 3) on the other hand, increases progressively with the continuation of the fast. The reasons for this are quite obvious. The drain put upon the reserve glycogen of the body due to fasting, and the forced elimination of a certain amount of glycocoll, increase the protein catabolism.

TABLE 4

PERIOD	DATE	WEIGHT	TOTAL N	HIPPURIC ACID N
<i>Rabbit 8</i>		<i>grams</i>	<i>grams</i>	<i>gram</i>
Fasting period	February 17	1950	1 442	0 201
	February 18	1850	1 806	0 120
Benzoic acid 1.25 grams per kilo	February 19	1750	2 100	0 112
	February 20	1650	1 988	0 096

In animals fed on dextrin the conduct of the total nitrogen and hippuric elimination, as observed above, and the absence of much, if any, food influence upon the latter, is even more strikingly illustrated.

Goetze and Pfeiffer<sup>1</sup> as well as Pfeiffer and Eber<sup>2</sup> noticed an increased hippuric acid output in the urine of horses, after feeding pentoses, and conclude from their observations that these animals eliminate most hippuric acid when their food is relatively rich in carbohydrates. A similar observation has been made on the human urine, after giving 100 grams of cane sugar and plenty of protein (cited from Wiechowksi). This is quite in keeping with our own experiments on the dextrin-fed rabbits.

From the work of most investigators, it seems that benzoic acid, by virtue of a toxic action, can disturb the general protein

<sup>1</sup> Goetze and Pfeiffer *Jahresber der Tierchem*, xvi, p. 804

<sup>2</sup> Pfeiffer and Eber *Ibid*, xxvii, p. 97-144

TABLE 5

TREATMENT	DATE	WEIGHT OF ANIMAL	FOOD			URINE		
			Dextrin	Oats	Carrots	Total N	Hippuric Acid N	Extra N
<i>Rabbit 8</i> I Control dextrin period	March 23	2050	grams 19	grams 10	grams 150	grams 0 375	grams 0 021	grams
	March 24	2050	19	40	150	0 343	0 017	
	March 25	2000	19	20	170	0 525	0 162	0 166
	March 26	1980	19	20	150	0 497	0 127	0 138
	March 27	1980	19	10	160	0 411	0 116	0 082
	March 28	2000	19	10	160	0 454	0 115	0 095
	April 17	2100	19	0	0	0 525	0 010	
	April 18	2080	19	0	0	0 406	0 015	
<i>Rabbit 10</i> I Control dextrin period	April 19	2005	19	0	0	0 399	0 014	
	April 20	2000	19	0	0	0 598	0 126	0 153
	April 21	2020	19	0	0	0 135	0 135	0 136
II Dextrin period plus benzoic acid 1.25 grams per kilo daily								

metabolism, but it appears that this action is exhibited only at certain times and under certain conditions which may or may not bear any relation to the glycocoll formation itself

Wiechowski<sup>1</sup> states that the gradual fall in the total nitrogen after prolonged feeding of benzoic acid is not due to an increased tolerance on the part of the animal for the benzoic acid. The toxic action of benzoic acid manifests itself in the loss of weight of the animal, and a decreased consumption of food. This, however, is not borne out by our own experiments on fasting and dextrin-fed rabbits, in the former the total nitrogen on the first day of the period is less than that on the corresponding day of the feeding period. We would naturally expect that the toxic action added to the increased catabolism produced by the fast, would cause an even greater disturbance in the protein decomposition—this, however, does not occur. Increased catabolism occurs apparently only (as evidenced by the daily increase in the total nitrogen) when the fasting is prolonged. Wiechowski evidently loses sight of the fact that the feeding of benzoic acid does not alone involve the protein catabolism, but that by removing available glycocoll from the body, it probably also produces a profound change upon the sugar, and hence the glycocoll production of the body, which in itself may cause a loss of body weight and increased nitrogen catabolism. The process is in all respects comparable to that obtained in phloridzin diabetes.

The dextrin-fed animals fail to show any toxic action of benzoic acid. They show no appreciable loss in weight, nor do they yield any excessive extra nitrogen apart from that required for the formation of hippuric acid, even on the first day of the benzoic acid period.

In the absence of any definite knowledge concerning the undetermined nitrogen in the urine on such days of benzoic acid feeding (when the extra nitrogen is greater than that of the glycocoll quota in hippuric acid), we can not say that a toxic decomposition of protein takes place. Ringer's results and our own, on urea and ammonia nitrogen outputs, indicate that the real body catabolism proceeds normally, notwithstanding the action of benzoic acid upon it. The whole trend of the experiments, especially those on the dextrin-fed animals, seems to point to the fact that

<sup>1</sup> Wiechowski *loc cit*

only the glycoll-bearing or glycoll-producing radicals are affected by the benzoic acid

It seems also that the animal body can become accustomed to the presence in it of benzoic acid and catabolize more protein, only to that extent to which the presence of a certain amount of benzoic acid at a given time compels the production of hippuric acid. Whether this tolerance for benzoic acid is in part attributable to an increased efficiency on the part of the excretories to eliminate free benzoic acid, we do not know. What is certain, however, is that even large doses of benzoic acid given at a certain rate, can not after a brief space of time cause any greater cleavage of protein than that necessary for the production of a certain amount of hippuric acid.

When, therefore, the benzoic acid feeding is extended over a prolonged period, the hippuric acid production reaches a definite level at which it continues. This level, however, is such that the extra nitrogen appearing in the urine is chiefly (at times entirely) in the form of hippuric acid. It is fair to assume from this that in other respects the protein metabolism, as evidenced by the difference nitrogen (that is total nitrogen minus hippuric nitrogen) remains undisturbed.

In his paper on the origin of glycoll, Ringer<sup>1</sup> suggests in conclusion that the increased glycoll output in the form of hippuric acid occurring after benzoate administration, "does not come from the fraction of protein that would have been metabolized had no benzoate been given, but entirely from the extra protein which is destroyed, due to the presence of a toxic substance."

From the evidence at hand, we would not regard the process involved in increased production of hippuric acid as toxic disintegration of protein with consequent production of glycoll. We would rather regard the action of benzoic acid in the formation of hippuric acid as a selective one and a compulsory one upon the liberation of glycoll-bearing radicals or its formation from simpler bodies by synthesis. The sulphur determinations substantiate this fact. Rather marked fluctuations in the total sulphur content of the urine in the normal periods occur under ordinary conditions of feeding, but experiments on fasting animals and those fed on dextrin, however, afford more favorable conditions for comparison.

<sup>1</sup> Ringer *loc cit*

TABLE 6

DATE	PERIOD	CONDITION	BF <sub>2</sub> SO <sub>3</sub> IC ACID (GPR KILO)	TOTAL N	DAILY AVERAGE N	IMIPURIC ACID N	DAILY AVERAGE IMIPURIC ACID N	TOTAL SULPHUR	DAILY AVERAGE TOTAL SULPHUR
<i>Rabbit 8</i>			grams	grams	gram	gram	gram	gram	gram
February 10	I	Feeding	0	1 372	1 067	0 053	0 032	0 161	0 152
February 11			0	1 155		0 030		0 153	
February 12			0	1 001		0 014		0 156	
February 13			0	0 742		0 026		0 141	
February 14	II	Feeding	1 25	1 573	1 476	0 193	0 175	0 178	0 156
February 15			1 25	1 491		0 184		0 126	
February 16			1 25	1 365		0 150		0 163	
February 17			1 25	1 442		0 201		0 147	
February 18	III	Fasting	1 25	1 806	1 931	0 120	0 132	0 162	0 159
February 19			1 25	2 100		0 112		0 171	
February 20			1 25	1 988		0 096		0 152	
February 21			1 25	1 358		0 125		0 171	
February 22	IV	Feeding	1 25	1 050	1 199	0 157	0 152	0 147	0 161?
February 23			1 25	1 190		0 145			
February 24			1 25	1 197		0 179			
February 25			1 25	1 299		0 140		0 186	
February 26	V	Fasting	1 25	1 981	1 640	0 182	0 161	0 163	0 168?
February 27			1 25					urine	
								lost	
								0 116	
February 28	VI	Feeding	1 25	1 804	1 216	0 097	0 150	0 146	0 161
March 1			1 25	0 945		0 174		0 179	
March 2			1 25	0 840		0 179		0 159	

The above experiments support the conclusion that there is no parallel between the protein metabolism and the glycocoll output in the form of hippuric acid. On comparing the two normal benzoic acid periods, we find that in the second, the total nitrogen is considerably lower, but its decrease is proportionately greater than the diminution in glycocoll, in the corresponding period, would lead us to expect. Likewise the total nitrogen in the first fasting benzoic acid period is greater in amount than that in the second, whereas the hippuric acid output is higher in the second fasting period.

It would seem from this, that the toxic action of benzoic acid is quite distinct from its power to produce the elimination of glycocoll. The former manifests itself only by changes in the amount of extra nitrogen eliminated at the beginning of each benzoic acid period—an effect which quickly wears off when the administration of the same dose of benzoic acid is continued.

However, if we regard the figures obtained in the sulphur of the different periods as a further indication of the process involved in protein metabolism, we must come to the conclusion previously reached, namely, that the benzoic acid does not cause much disturbance in the general protein metabolism. The toxic action manifested at the inception of each period is more apparent than real. The action of benzoic acid is exerted preferentially upon glycocoll radicals whose production it evidently can force either by hydrolysis or by synthesis, without effecting further change in the decomposition of protein.

The apparent independence of the process involved in the production of glycocoll for the formation of hippuric acid from the rest of the protein catabolism is supported by the data obtained in experiments with pure carbohydrate feeding.

The results in the above experiments are very striking in the precision with which they demonstrate the manner of the glycocoll production and the rôle it plays in protein metabolism. Two facts appear very plainly, namely, the effect of the dextrin on the protein metabolism as a whole, and the relation it bears to the production of glycocoll from protein in response to benzoic acid. In the above tables will be seen the sparing of protein catabolism and the consequent fall in the nitrogen output caused by the dextrin feeding. Notwithstanding this diminution, the amount of glycocoll on feeding benzoic acid during the dextrin periods is

TABLE 7

DATE	PERIOD	FEEDING	DEXTRIN	BENZOIC ACID (PER MLO)	TOTAL N	DAILY ALPHA TOTAL N	HIPURIC ACID N	DAILY ALPHA HIPURIC ACID N	TOTAL SUFFIC	DAILY AVERAGE TOTAL S
<i>Rabbit 8</i>										
March 11	I	Normal	0	0	1 057	0 731	0 013	0 018	0 234	0 210
March 12			0	0	0 749		0 022		0 235	
March 13			0	0	0 539		0 021		0 237	
March 14			0	0	0 581		0 019		0 232	
March 15	II	Normal	0	1 25	0 805	1 016	0 140	0 126	0 218	0 205
March 16			0	1 25	1 143		0 106		0 208	
March 17			0	1 25	1 090		0 133		0 190	
March 18			0	0	0 658		0 011		0 203	
March 19	III	Normal	0	0	lost	0 626	0 019	0 018	0 186	0 195
March 20			0	0	0 560					
March 21			0	0	0 665		0 025		0 195	
March 22			19	0	0 385		0 015		0 171	
March 23	IV	Normal	19	0	0 375	0 364	0 021	0 018	0 152	0 163
March 24			19	0	0 343		0 017		0 165	
March 25			19	1 25	0 525		0 162		0 162	
March 26			19	1 25	0 497		0 127		0 144	
March 27	V	Normal	19	1 25	0 441	0 479	0 146	0 145	0 164	0 157
March 28			19	1 25	0 454		0 145		0 159	

TABLE 7—Continued

DATE	PERIOD	FEEDING	DEXTIN	BENZOIC ACID (PER 100)	TOTAL N	DAILY AVERAGE TOTAL N	HIPPURIC ACID N	DAILY AVERAGE HIPURIC ACID N	TOTAL SULPHUR	DAILY AVERAGE TOTAL S
				grams	grams	grams	gram	gram	gram	gram
<i>Rabbit 10</i>										
April 8	I	Normal	0	0	0 763	0 012	0 012	0 012	0 184	0 181
April 9			0	0	0 777	0 012	0 012	0 012	0 188	0 181
April 10			0	0	0 854	0 012	0 012	0 012	0 180	0 181
April 11			0	1 25	1 519	0 090	0 090	0 090	0 182	0 180
April 12	II	Normal	0	1 25	1 403	0 126	0 126	0 126	0 174	0 180
April 13			0	1 25	1 169	0 169	0 169	0 169	0 181	0 180
April 14			0	0	0 791	0 011	0 011	0 011	0 108	0 180
April 15			0	0	0 546	0 010	0 010	0 010	0 178	0 180
April 16	IV	None	0	0	0 525	0 013	0 013	0 013	0 116	0 133
April 17			19	0	0 525	0 010	0 010	0 010	0 147	0 133
April 18			19	0	0 406	0 015	0 015	0 015	0 148	0 133
April 19			19	0	0 399	0 014	0 014	0 014	0 150	0 157
April 20	V	None	19	1 25	0 592	0 126	0 126	0 126	0 150	0 157
April 21			19	1 25	0 591	0 136	0 136	0 136	0 154	0 157



greater than that found in the urine in the periods of normal feeding

In the first of these experiments (rabbit 8, table 7) the total nitrogen of the dextrin-benzoic period (V) is less than half of the preceding control-benzoic period (II), that is, 0.479 gram as compared with 1.016 grams. The glycocoll nitrogen output for the corresponding periods is 0.145 gram or 30.3 per cent of the total, as compared with 0.126 gram or 12.4 per cent.

The figures in the second experiment (rabbit 10, table 7) for total nitrogen and hippuric acid nitrogen are even more instructive. The total nitrogen in the dextrin-benzoic period (V) is a little over 40 per cent of the preceding control benzoic period (II), and yet of that (0.586 gram) 0.131 gram or 22 per cent is glycocoll nitrogen as compared with 0.128 gram (out of 1.383 grams N) or 9 per cent of the preceding period.

It should be noted, however, that notwithstanding the decrease in the total nitrogen of both dextrin-benzoic acid periods (rabbit 8, table 7, V) and (rabbit 10, table 7, V), the extra nitrogen eliminated is in each instance covered by the glycocoll nitrogen.

In regard to the sulphur output in all these experiments, it seems that it is subject to variation, depending upon the sulphur content of the food consumed, under like conditions of experimentation, remaining fairly constant. It is evidently not affected by the benzoic acid itself—which is a further proof that benzoic acid exerts its action on none but the glycocoll-producing elements of body protein.

We have already seen that the glycocoll output by the fasting animal (rabbit 8, table 7) during benzoic acid feeding is, comparatively speaking, high, though not quite as high as in the control periods. The extra nitrogen eliminated at the same time is considerably more than that present in the glycocoll, and increases as the fast progresses. In experiments on animals fed on dextrin and benzoic acid (rabbit 8, table 7, and rabbit 10, table 7) all the extra nitrogen appears in the form of glycocoll. If we compare these results, we are justified in drawing the conclusion that the glycocoll being withdrawn from the body in starvation, an extra amount of protein is catabolized. In the dextrin-fed animals, the glycocoll is not required as a source of energy, and all available glycocoll is removed by benzoic acid without affecting the rest of

protein metabolism in any way. The data obtained by sulphur determinations are in accord with this conclusion.

Thus we have an indication of the unique formation of glycocoll and of the sparing effect of carbohydrates upon protein. Our present knowledge of the formation of sugar from amino-acids (more definitely glycocoll), leads us to assume that the "dynamic quota" (Rubner) of protein in metabolism is furnished by the amino-acids through a primary conversion into sugar. Liberal carbohydrate feeding makes this source of production unnecessary and the amino-acids, that is the glycogenetic radicals, remain intact.

Whatever our views of the structure of protein may be, the fact remains that under certain conditions the animal body may be forced to produce a large amount of glycocoll which is in excess of that known to be present preformed in any protein, and furthermore, that this excessive production of glycocoll need not be accompanied by any evidence of an increased cleavage of any other protein radicals.

The question which this fact suggests, is whether or not the hydrolytic process by which glycocoll is formed *in vitro* is comparable to the catabolic process involved in its production in the body. It seems not. It is possible that the hydrolysis of protein *in vivo* is far more complex than that occurring *in vitro*, and that more than 3 per cent of glycocoll exists preformed in tissue protein. This would readily explain the increased production of glycocoll when benzoic acid is administered. It would also explain the origin of glycocoll in the tissue proteins of young animals fed on protein materials poor in glycocoll. Otherwise we must assume that glycocoll can be synthesized in the body from still simpler substances. Spiro conceives of the possibility of synthesis of glycocoll out of  $-\text{CH}_2-\text{COOH}$  and  $\text{NH}_2$  radicals. Whereas Parker and Lusk suggest the synthesis of glycocoll from  $\text{CH}_3\text{COOH}$  and  $\text{NH}_3$ . Wiechowski argues against such a process on the basis that glycocoll is eliminated in large amounts in starvation. Our own work leads us indirectly to the belief, however, that such a process is not at all unlikely and may be responsible at least in part, for the excessive glycocoll production on feeding benzoic acid. The third and other possibility of the origin of glycocoll is the decomposition of higher amino-acids under the influence of ben-

zoic acid circulating in the blood Magnus-Levy showed that benzoyl-leucine can be converted into hippuric acid Though this experiment in itself is not absolutely conclusive, it argues in favor of the formation of glycocoll from higher amino-acids through a primary benzoylization

It seems not unlikely from the data obtained in our work that glycocoll can be split off from the tissue proteins independently of the destruction of the rest of the protein molecule, when the needs of the body require it The possibility of such dissociation is not without analogy However, in all known conditions in which leucine appears in the urine, we find other evidences of disturbed and usually increased protein destruction In phosphorus poisoning and in acute yellow atrophy of the liver, the presence of leucine in the urine is associated with other amino-acids and other protein radicals, but in the increased production of glycocoll as hippuric acid on feeding benzoic acid, we find no sufficient evidence of any increased or abnormal protein destruction In other words, the production of glycocoll is not a toxic process and is comparable in all respects, to the production of cystine—a substance which evidently can occur in the urine without being associated with any other manifestations of disturbed protein metabolism

#### CONCLUSIONS

To summarize briefly, our results indicate

1 That no "reserve" of glycocoll exists in the animal body The production of glycocoll is progressive, depending, to a certain extent, upon the amount of benzoic acid present in the circulation

2 That benzoic acid does not exert a truly toxic action on protein metabolism, that is to say, it does not cause a massive decomposition of proteid

3 That the action of benzoic acid in the production of hippuric acid in the body is a selective one, it combines directly with glycocoll, or with such other radicals which can ultimately be changed into glycocoll The process here involved may be a destructive one of higher amino-acids, or a constructive one from simpler bodies In other respects the protein metabolism progresses normally

4 That other protein radicals are unaffected by benzoic acid, beyond that necessary to replace the glycocoll eliminated as hippuric acid

5 That the sparing effect of carbohydrates on proteids is affected by removing the necessity for the production of a "dynamic quota" from the glycogenetic radicals of proteid

6 In carbohydrate feeding the extra nitrogen eliminated in consequence of benzoic acid administration, is entirely in the form of hippuric acid, so that the production of glycocoll for the formation of hippuric acid can occur independently of the rest of protein metabolism

We desire to thank Dr Theodore Kuttner, of this Laboratory, for his assistance in some of the analytical work



# ON A METHOD FOR THE PREPARATION OF NUCLEIC ACID

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(Received for publication, October 16, 1911)

This method differs from others in the use of barium hydroxide as the alkali which (with sodium chloride) is used for the extraction of the tissue. The alkalinity thus obtained is sufficient to decompose the nucleoproteins. The distinctive advantage of the method is the comparative insolubility of the barium compounds formed with constituents of the tissue. So little of the protein enters into solution that a separate precipitation of the proteins is not made. This is a necessary preliminary in all other methods and its omission here avoids a troublesome filtration. The barium hydroxide-sodium chloride solution dissolves nucleic acid freely. Tests show that both barium and proteins are effectually excluded from the final product. Guanylic acid owing to its ready solubility in dilute hydrochloric acid is also absent.

## I THE METHOD OF PREPARATION

The tissue is passed through a meat grinder and mixed with a half-saturated solution of common salt in the proportion of one to two. Powdered barium hydroxide, sufficient to maintain a distinctly alkaline reaction to litmus paper, is added at intervals while heating. The amount of barium hydroxide necessary for this purpose was found to range from 0.5 to 1.0 per cent of the weight of the moist tissue if taken fresh. One kilo of fresh cow's liver required about 5 grams, of fresh pig's liver, 7.5 grams. An older but still edible cow's liver required 7 grams but a kilo of the same liver after keeping twenty-four hours at 38–40° C. required 24 grams. An indiscriminate excess of barium hydroxide was used in the earlier experiments but this results in a modification

of the properties of the nucleic acid. When the boiling point has been reached, but not sooner, the concentration of salt is reduced to about one-third saturation by the addition of distilled water. Alkalinity to litmus paper must be maintained. Heating (just below boiling) is continued for a half-hour without replacing evaporated water. The hot mixture is strained through a filtering bag or filtered by suction through a Buchner funnel in order to remove the greater part of the undissolved material.

The extract when thoroughly cold is sufficiently acidified with 20 per cent hydrochloric acid to produce abundant precipitation. Continuous stirring is necessary while the acid is being added. The mixture is shaken or stirred with some ether to increase the flocculation of the precipitate and to facilitate the following filtration. It is filtered through Buchner funnels, (S & S No 597 filters used double, if necessary) the filter having been previously moistened with saturated salt solution. If turbid the filtrates are returned to the filters. Persistent turbidity is obviated by the addition of saturated salt solution. The precipitate should not remain long in contact with the acid liquid but it may be allowed to settle before the filtration. Washing is not necessary but the filters should be drained by suction.

The filters with precipitates are heated in a porcelain evaporating dish with a solution consisting of equal volumes of pure saturated sodium chloride and of normal sodium hydroxide, while continuously stirring. The filter paper should be disintegrated by stirring but not removed. After cooling the mixture six-tenths of its volume of 96 per cent alcohol is added and the whole is allowed to stand a short time. It is then filtered through a Buchner funnel carrying a filter (S & S No 597 was used) moistened with alcohol. In order to obtain a final product free from heavy impurities this filtrate must be *absolutely* clear. If it still shows a slight turbidity further filtration usually fails to clear it but if it is allowed to stand over night in a conical beaker a perfectly clear liquid can then be decanted.

The above treatment dissolves the nucleic acid and leaves glycogen and other polysaccharides as an insoluble residue. As is well known,<sup>1</sup> an alkaline solution of glycogen is readily precipitated

<sup>1</sup> Hoppe-Seyler-Thierfelder *Handb d chem Analyse*, 8<sup>te</sup> Aufl, p 122, 1909

by a moderate addition of alcohol and still more so from a solution containing much sodium chloride. In the above solution the concentration of sodium chloride after adding the alcohol is practically one-third of saturation. The conditions are thus favorable for the removal of polysaccharides, only a limited quantity of which could enter the original barium hydroxide + sodium chloride extract. If any fats and lipoids were dissolved in the original extract they are still present as they have the same solubility as nucleic acid in the menstrua thus far used. The liquid still gives a strong biuret reaction although the bulk of the tissue proteins remained in the residue insoluble in the solution of barium hydroxide plus sodium chloride.

The clear liquid containing the nucleic acid is now transferred to a stoppered separatory funnel. Some saturated solution of pure sodium chloride is added and then a layer of ether from one-half to one centimeter in depth. Twenty per cent hydrochloric acid is then added to precipitate the nucleic acid. The funnel is thoroughly shaken and allowed to stand a few minutes. If the quantity of ether and of sodium chloride is sufficient the nucleic acid will be carried mechanically to the top in the ethereal layer. If the separation into two layers does not occur readily more solution of sodium chloride and more ether are added and the shaking is repeated. The lower layer of liquid is withdrawn and discarded. The mixture of ether and nucleic acid remaining in the funnel is washed out of it with 60 per cent alcohol and is transferred to a conical beaker. A small Buchner funnel is prepared with two filters, the lower one being an S & S No. 589 and the upper one a hardened filter. They are first moistened with strong alcohol, then the ethereal layer is poured on from the beaker and finally the 60 per cent alcoholic mixture containing most of the nucleic acid precipitate. The precipitate on the filter is washed successively with 60 per cent alcohol, strong alcohol and finally with dry ether. As long as the liquid carrying the precipitate consists chiefly of ether or alcohol the filter paper will not become stopped. If the first filtrate that runs through turbid is returned the filter will soon retain all the precipitate. A light white and porous powder should remain on the filter. It should be transferred to a wide flat-bottomed dish which is placed in a desiccator over sulphuric acid *in vacuo*. If the layer of nucleic acid on the filter paper is



gelatinous and sticky the above processes by which water and salt are removed have not been thoroughly performed. The product was always kept several hours in the desiccator before it was weighed for analytical purposes.

The "funnel process" described above is an essential feature of this method. The substitution of simple filtration for it results in a poorer product as shown by analysis. The separation of the nucleic acid by means of the separatory funnel offers less favorable conditions for the adsorption of foreign substances by the flocculent nucleic acid. The rejection of the lower layer serves the important purposes of removing most of the sodium chloride, all other impurities that are not precipitated by the acid in the presence of salt, and also the substances which give the biuret reaction. These latter are a source of much difficulty in the preparation of pure nucleic acid and they are here separated by means of their ready solubility in dilute alcohol in the presence of sodium chloride. The fats and lipoids which have accompanied the nucleic acid from the beginning are still held in solution by the alcohol-ether and are removed in the final filtration and washing.

In order that the method may yield the purest product as judged by the content of phosphoric acid the sample of nucleic acid first obtained should be subjected to a repetition of part of the above procedure. This second treatment may begin with the solution of the specimen in sodium hydroxide plus sodium chloride as previously described.

The method of extracting the original tissue with barium hydroxide plus sodium chloride is practically quantitative if the first solid residue is heated a second time with half saturated salt solution and a little barium hydroxide. Further repetitions of the extraction carry substances into solution but when the "funnel process" is reached show practically no nucleic acid.

## II THE IDENTITY OF THE SUBSTANCE

In the development of this method the percentage of phosphorus in the nucleic acid obtained by any given procedure was regarded as an important criterion of its success. In the following table are given the results of determinations of phosphorus and nitrogen in specimens of nucleic acid obtained after the method had

been developed as above described. The nitrogen was determined by the Kjeldahl method, the phosphorus by the volumetric method of A. Neumann<sup>1</sup>

*Determinations of Phosphorus and Nitrogen in Nucleic Acids*

I *Nucleic acid from liver of pig*

NO	WEIGHT OF NUCLEIC ACID	WEIGHT OF PHOSPHORUS	PER CENT OF PHOSPHORUS	WEIGHT OF NUCLEIC ACID	WEIGHT OF NITROGEN	PERCENT OF NITROGEN
	mgms	mgms		mgms	mgms	
42 (4)	31.8	2.66	8.36			
48 (1)	33.3	2.84	8.51	57.8	8.06	13.94
48 (2)	34.8	3.07	8.82	83.5	12.76	15.28

II *Nucleic acid from liver of cow*

43 (1)	43.2	3.55	8.21			
47 (1)	31.5	2.60	8.26			
50 (2)	32.7	2.71	8.30			
51 (1)	30.6	2.83	9.32	85.0	12.79	15.05

III *Nucleic acid from liver of cat*

58	24.6	2.02	8.19			
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IV *Nucleic acid from spleen of pig*

54				76.6	11.24	14.67
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The pig and cow livers and the spleen used in obtaining the above data were received within twenty-four hours after slaughtering and had been kept in refrigeration during that time. However, the cow liver No. 50 (2) was divided into two portions, the first of which was immediately used but the second portion, which was numbered 51 (1) was kept in a warm room for twenty-four hours at a temperature of 38° to 40° C. It then smelled strongly acid and 24 grams of barium hydroxide were required to keep the tissue alkaline during extraction, the first portion having required only 8 grams of barium hydroxide. The weight of liver or spleen used in one operation varied from 1 to 10 kgm. No. 58 was a portion of the nucleic acid obtained from eleven cats, the fresh livers weighing not less than 1100 grams.

<sup>1</sup> A. Neumann, *Einfache Veraschungsmethode (Säuregemisch-Veraschung)*, *Zeitschr. f. physiol. Chem.*, **xxvii**, pp. 115-142, 1902-03, *Nachträge zur Säuregemisch-Veraschung und zu den an diese angeknüpften Bestimmungsmethoden*, *Zeitschr. f. physiol. Chem.*, **xlii**, pp. 32-36, 1904-05.

The results show a considerable degree of uniformity indicating an average 8-9 per cent of phosphorus and 14-15 per cent of nitrogen. These numbers were obtained upon small weights of nucleic acid and consequently the figures of the table are accurate only to within certain limits of error. They are presented mainly as indication of the identity and the quality of the nucleic acid. Besides the quantitative determinations of phosphorus and nitrogen qualitative examination was made with positive result for the characteristic constituents of nucleic acid.<sup>1</sup>

The final product could always be purified so that it failed to give the biuret reaction. This reaction was performed with copper chloride instead of sulphate. The former salt gives a much more sensitive reaction. There were occasionally specimens of nucleic acid that were sufficiently protein-free not to give the reaction with the sulphate but which gave a positive result with the chloride. Furthermore the reaction was performed by keeping the alkaline solution of nucleic acid and the copper chloride solution in adjacent strata for the examination of a biuret ring and finally mixing the whole. To detect colors the test tube (of about 1.5 cm in diameter) was placed in the hollow of a white porcelain evaporating dish held vertically on a level with the eye, the observer having his back towards the source of daylight.

The carbohydrate reactions with phloroglucin and orcin<sup>2</sup> were obtained with all the specimens tested with these reagents. The characteristic colors appeared without a precipitate before the addition of amyl alcohol and the latter agent readily extracted the coloring matter. According to Steudel,<sup>3</sup> in the case of nucleic acid the coloring matter should not be extractable by amyl alcohol. It may be added that by hydrolysis no substance having a reducing action upon alkaline copper solution could be obtained although the extracts of liver in the first parts of the procedure had reducing power. The absence of reducing power from the final product agrees with the observations of Bang<sup>4</sup> and Kossel on nucleic acid.

<sup>1</sup> Hoppe-Seyler-Thierfelder *Handb d chem Anal*, 8<sup>te</sup> Aufl, §§436, 439

<sup>2</sup> Hoppe-Seyler-Thierfelder *Handbuch d chem Analyse*, 8<sup>te</sup> Aufl, §§ 103, 439 with footnote

<sup>3</sup> H. Steudel Über die Kohhydratgruppe in der Nucleinsäure, *Zeitschr f physiol Chem*, LV, p 215

<sup>4</sup> Ivar Bang Chemische Untersuchungen der lymphatischen Organe, *Hofmeister's Beiträge*, XV, p 341, 1904

With reference to the persistence of barium in the final product it was observed that after hydrolysis with sulphuric and nitric acids as in Neumann's method for the volumetric determination of phosphorus, or after hydrolysis with sulphuric acid alone, not the least trace of precipitate appeared upon dilution of the hydrolyzed liquid. Chlorine also was absent from the boiled nitric acid solution of the nucleic acid.

This method was developed by means of experiments on liver. This is unfavorable material for the production of pure nucleic acid on account of the large proportion of carbohydrate and other foreign substances. Having found it workable on this tissue no difficulty was found in its application to other glands, *e g*, spleen, that are more commonly used as sources of nucleic acid. After the numerous experiments that were found necessary to develop the method to a stage of usefulness no time remained for the determination of the approximate percentage yield of nucleic acid for a given weight of tissue. A rough estimation would place this yield at less than one-tenth per cent of the moist weight.

With much pleasure and gratitude I acknowledge my great obligation to Professor Otto Fohn for the use of laboratory facilities and materials and for his kindly encouragement of this work.



CONTRIBUTIONS TO THE PHYSIOLOGY OF LYMPH  
XVIII THE RELATION OF THE PANCREAS TO THE LIPASE OF THE  
BLOOD AND THE LYMPHS

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(Received for publication, October 27, 1911)

INTRODUCTION

The primary aim in these experiments was to determine more definitely the factors concerned in the maintenance of the normal concentration of the lipase of the blood and the lymph. In the work on blood and lymph diastases in this laboratory by Carlson and his associates the results, at least for the dog, point to the importance of the internal secretion of the pancreas in the maintenance of the normal diastase concentration<sup>1</sup>. In view of these facts a reinvestigation of the relation of the blood and lymph lipase to the external and internal secretions of the pancreas seemed urgent and this was undertaken at Professor Carlson's suggestion. There is no doubt that under certain experimental conditions pancreatic lipase, as well as pancreatic amylase, enters the blood. Nevertheless it would seem from the present work that the factors governing the diastase and lipase concentrations in the blood are either different or differently adjusted, for, under experimental conditions, especially in relation to the pancreas, the lipase concentration is not subjected to the wide variations that occur in the diastases.

I LITERATURE

The work on the lipases of the body tissues is reviewed by Connstein<sup>2</sup> and especially by C. Oppenheimer<sup>3</sup>.

<sup>1</sup> Carlson and Luckhardt *Amer Journ of Physiol*, xxiii, p 148, 1903, Otten and Galloway *ibid*, xxvi, p 347, 1910, Gould and Carlson *ibid*, xxix, 1911.

<sup>2</sup> Connstein *Ergeb d Physiol*, iii, p 194, 1904.

<sup>3</sup> Oppenheimer *Die Fermente*, 1909.

*The nature of the serolipase* It is to be noted that most of the work on blood lipases has been with artificial esters and not true fats. Even Hanriot<sup>1</sup> says, that fats, while acted upon by the blood, are not suited for the study of hydrolysis because of their insolubility and small amount of cleavage. In their place he used a fatty acid ester, monobutyrin. This is more easily emulsified and split by the blood serum and therefore better adapted for following the course of hydrolysis. Kastle and Loevenhart<sup>2</sup> and Loevenhart<sup>3</sup> also laid stress upon the similarity of the action of lipase upon ethylbutyrate and fats.

Following this a heated discussion arose concerning the lipases of the serum. Arthus<sup>4</sup> and Doyen and Morel<sup>5</sup> claim that blood serum does not contain a true lipase but only esterases of the lower fatty acids. In 1904 Bitnn-Schlachto<sup>6</sup> found that serolipase splits artificial fats as easily as monobutyrin. It may be granted that esterases resemble enzymes in general, but the question now arises whether or not these are identical with true lipases. The facts favoring their identity may be stated as follows:

1 Fats and ethereal salts are analogous chemically and are hydrolyzed in the same way.

2 Both have been synthesized by lipase. Thus, Kastle and Loevenhart,<sup>7</sup> Hanriot,<sup>8</sup> Acree and Hinkins,<sup>9</sup> and Bodenstein,<sup>10</sup> have formed various esters from their corresponding constituents. The synthesis of both natural and higher fats was demonstrated by Pottevin.<sup>11</sup>

3 Pancreatic juice splits both fats and esters. Hewlett<sup>12</sup>

<sup>1</sup> Hanriot *Compt rend acad d sci*, cxviii, p 753, 1896

<sup>2</sup> Kastle and Loevenhart *Amer Chem Journ*, xiv, p 491, 1900

<sup>3</sup> Loevenhart *Amer Journ of Physiol*, vi, p 331, 1902

<sup>4</sup> Arthus *Journ physiol et path*, iv, p 455, 1902

<sup>5</sup> Doyen and Morel *Compt rend soc biol*, lv, p 682, *Journ physiol et path*, iv, 1902

<sup>6</sup> Bitnn-Schlachto *St Petersburg Diss*, 1904, cited from *Biochem Centralbl*, iii, p 24, 1904

<sup>7</sup> Kastle and Loevenhart *Loc cit*

<sup>8</sup> Hanriot *Compt rend acad d sci*, cxviii, p 212, 1900

<sup>9</sup> Acree and Hinkins *Amer Chem Journ*, xxviii, p 370, 1902

<sup>10</sup> Bodenstein *Zeitschr f Electrochem*, vii, p 605, 1905

<sup>11</sup> Pottevin *Ann Inst Pasteur*, x, p 901, 1906

<sup>12</sup> Hewlett, *Journ Med Research*, vi, p 377, 1904

showed that, after pancreatitis, lipases are absorbed by the blood and excreted in the urine where they are capable of acting upon both ethylbutyrate and olive oil

4 These foregoing comparisons are based upon the titrations of the acid formed in hydrolysis Saxl<sup>1</sup> does not think this method admits of quantitative comparisons of lipase values In his work he boiled and filtered his tests just before titration By this means the coagulable proteins are removed, the presence of which, he says, gives errors in actual lipase values According to his results fats are not split at all by the various body fluids, while esters are affected only to a slight degree, much less than when the tests are not boiled Saxl's results, however, are open to two objections

(a) The lipase is inhibited through the addition of too much toluol (Oppenheimer)<sup>2</sup> A concentration of 6 per cent was used where Kastle and Loevenhart employed 2 per cent

(b) Boiling diminishes the amount of total acid present, for, if to diluted serum a known amount of butyric acid be added, the solution divided into two equal parts and one boiled and filtered, the latter loses a share of its acidity as compared with the unboiled control This in turn titrates its full acid content plus the original acidity The objection, that the potential acidity of these coagulable proteins present interferes with the titration of the hydrolyzed acid, may be met by neutralizing the tests immediately before incubation

Quite recently a new method was introduced in which the amount of zymolyte remaining after a given time of incubation was determined by the change of the solution tension of the mixture (Rona and Michaelis)<sup>3</sup> Serum of horses, cats, sheep and cattle was found to have similar action upon both monobutyrin and tributyrin

From the analogies above mentioned there seems to be no doubt that the esterases are true lipases and that their action is qualitatively directly applicable to the hydrolysis of natural fats

*The source of the blood lipase* Hanriot<sup>4</sup> was the first to inves-

<sup>1</sup> Saxl *Biochem Zeitschr*, **xii**, p 343, 1908

<sup>2</sup> Oppenheimer *Loc cit*

<sup>3</sup> Rona and Michaelis *Biochem Zeitschr*, **xxx**, p 345, 1911

<sup>4</sup> Hanriot *Compt rend acad sci*, **cxxiii**, p 803, 1896



tigate the origin of serolipase. After extirpation of the pancreas in a dog he found on the following day the activity of the serum increased to three times the normal. However, an autopsy showed that two pieces of the gland still remained and hence the results is not conclusive. Later, he proved<sup>1</sup> that the lipases of the pancreas and serum act differently in their speeds of hydrolysis (a) with previous neutralization by sodium carbonate, and (b) at different temperatures (15° and 42°). In 1902 Loevenhart<sup>2</sup> pointed out similar and other differences between those of liver and pancreatic extracts and concluded that on the one hand the lipases of the blood, liver and kidneys are alike, while on the other hand the lipases of the pancreas and remaining tissues are the same. Two years later Bitnu-Schlachto<sup>3</sup> found that lipases of bone marrow and serum acted differently.

That the lipase of the serum does not come from the destruction of the leucocytes on drawing the blood was demonstrated by Hanriot,<sup>4</sup> who found plasma and serum possessed the same lipolytic activity. The most recent work found in the literature on lipase in relation to the pancreas is by Hewlett<sup>5</sup> who points out that, in lesions of this organ, lipase passes into the blood and is excreted by the kidneys.

## II METHODS

1 *Preparation of the serum* Dogs were bled from the external saphenous vein or the heart. The blood of rabbits was obtained by cutting the marginal vein of an ear. That of pigs was taken from the ear or the tail. All blood was defibrinated, centrifuged one and one-half to two hours and immediately tested.

2 *Method of testing the lipolytic power* Since the action of serum upon the lower esters, as ethylbutyrate, may be taken as similar to the action on natural fats, Kastle and Loevenhart's method<sup>6</sup> was tried first. It was found that lipase in dog's serum

<sup>1</sup> Hanriot *Compt rend soc biol*, p 778, 1897

<sup>2</sup> Loevenhart *Loc cit*

<sup>3</sup> Bitnu-Schlachto *Loc cit*

<sup>4</sup> Hanriot *Compt rend acad sci*, cxviii, p 853, 1896

<sup>5</sup> Hewlett *Loc cit*

<sup>6</sup> Kastle and Loevenhart *Loc cit*

is present in very small amounts so that it became necessary to increase the extent of hydrolysis. Besides, the serum often contained traces of haemoglobin which obscured the end point. Other factors also had to be considered, for, by increasing the time element, inhibition of hydrolysis entered in through the accumulation of end-products. Uncertain weather was an obstacle and on dark days it became extremely difficult to determine the end of titration. By increasing the volume of the mixture from 5 to 25 cc it was found that the color of the haemoglobin was practically eliminated, end-products were kept at a minimum dilution and inhibition of the enzyme thereby prevented. The ethylbutyrate was made at a 5 per cent concentration in order to keep it maximal. Incubation was lengthened to four hours. Reading by sunlight was discarded and a tungsten filament used. This was enclosed in a suitable box in the upper side of which two circular openings were cut and fitted with white ground glass.

The method finally adopted was as follows

Into each of three 60 cc Erlenmeyer flasks, A, B and C, were introduced 25 cc of water, 0.5 cc of toluol, and 0.1 cc of phenolphthalein (1 per cent), the flasks corked, shaken and heated to 37° C. One cubic centimeter of serum was then added to A and B, respectively, their contents neutralized with  $\frac{N}{20}$  sodium hydroxide and 1.25 cc of ethylbutyrate added. The flasks were tightly corked, thoroughly shaken a hundred times each to produce a complete emulsion and immediately placed in the thermostat. To C, 1 cc of serum (previously diluted and boiled) was added, the mixture neutralized, 1.25 cc of ethylbutyrate added, the flask tightly corked, shaken and also placed in the incubator. Other controls with ethylbutyrate and with serum respectively were used in many instances to determine any spontaneous hydrolysis. These were found negligible. After four hours the flasks were removed, cooled in tap water (4° to 8° C) and titrated with  $\frac{N}{20}$  sodium hydroxide. In contradiction to the reports of many previous workers the boiled controls were not found negative but always gave 0.20 to 0.25 cc hydrolysis for serum of all animals tested.

Pure ethylbutyrate, neutral to phenolphthalein was used (Mallinkrodt's). In titration, a 10 cc burette with a capillary dropping point, giving about thirty drops per cubic centimeter, permitted very close titrations to be made and, in trying out the accuracy of the method, it was found that the results invariably checked within 0.05 cc.

*S Conduct of experiments* Extirpation of the pancreas was made in dogs and rabbits. Ligation of pancreatic ducts and ligation of ducts and all pancreatic tissue adjacent to the duodenum

were tried in dogs, the aim being to follow at first the immediate effect of obstruction of the external secretion and later the result of atrophy of the gland. It was found, however, that, because of the close relation of the pancreas and the duodenum and of the numerous adhesions following operation, reestablishment of ducts tended to occur and degeneration of the gland thus prevented. To overcome this the middle third of the pancreas was removed in two dogs and the cut ends cauterized. More favorable conditions for the study of atrophy of the gland are offered in the rabbit. Here the pancreas is not closely associated with the gut and so is free from the obstacles presented in the dog.

From Hewlett's work<sup>1</sup> it seems evident that the kidneys in pancreatic lesions are a potential factor in lipase excretion. Hence the effect of elimination of urine secretion was determined, both separately and with pancreatic variations mentioned in the preceding paragraph. The result of increased elimination was also investigated.

Other glands of internal secretion, such as the thyroid, were studied in relation to the lipase of the blood under conditions of hyper- and hypo-thyroidism.

### III RESULTS

In the course of the experiments it was found that the daily lipolytic power of the serum in normal animals remained remarkably constant. Thus dog's serum showed a variation never greater than 0.20 cc, except in the summer when a range of 0.30 cc sometimes occurred. Rabbit's serum, which has a considerably higher lipolytic power, remained very uniform and only occasionally deviated either way. That such variations, though small, should occur is inevitable, if serolipase arises from cell lipases and particularly because the concentration of the blood is varied by water ingestion and other factors. In the diuresis experiments, where physiological salt solution was given by stomach tube, the lipolytic variation of the serum proved to be largely a matter of blood dilution.

Serolipolytic power in dogs ranged from 0.30 to 0.50 cc during the winter among some fifty animals examined, except in two which

<sup>1</sup> Hewlett *Loc cit*

gave 0.80 and 1.00 cc respectively. The range during the summer months was found to be at a higher level (0.40 to 0.80 cc). Even the same dogs showed an increase in hot weather. The concentration of lipase in rabbit's serum was not so constant for the species but varied from 0.75 to 4.00 cc among individuals. Pig's, sheep and goat's serum gave only a mere trace of lipase activity. On the other hand birds, such as the duck and the pigeon, showed tremendous activity, the duck testing over 11.00 cc and the pigeon, 7.00 cc.

### 1 *Pancreatectomy*

The pancreas was extirpated in nine dogs and one rabbit. In all the dogs the lipase curve showed no change, while in the rabbit only a temporary fall occurred. The diastases in many of the dogs were also followed and, to show the surprising contrast between the lipase and diastases of the serum under these conditions, the following typical experiment is given in detail.

TABLE I  
*Experiment 2 Dog Pancreas removed February 27, 1911*

DATE	LIPOLYTIC ACTIVITY*	DIASTATIC ACTIVITY†
February 23	0.33	1.45
February 25	0.35	1.45
February 28	0.40	2.30
March 3	0.37	2.25
March 9	0.30	3.30
April 16	0.30	4.30

\* In this and the following tables the lipolytic power is expressed in cubic centimeters of  $\frac{N}{10}$  NaOH required to neutralize the acid produced in four hours.

† In this and the following tables the diastatic power is given in hours. The diastases were tested by Mr. Gould. One cubic centimeter of serum was added to 20 cc. of 1 per cent starch solution, the mixture kept at 37° C. and tested for the disappearance of the blue starch reaction by means of a weak I-KI solution. The diastases in many of the subsequent experiments were tested by Dr. Van de Erve. In his method 1 cc. of serum was added to 10 cc. of 1 per cent starch solution and a 0.05 per cent I-KI solution used to determine the disappearance of the erythro-dextrin stage, which was taken as the end-point. As thus expressed the higher figures indicate diminution in the concentration of the serum diastases, while in the cases of the lipolytic power the higher figures indicate an increased concentration.

TABLE II  
Summary of pancreatectomy experiments

EXPERIMENT	NORMAL			AFTER PANCREATECTOMY				
	Day*	Lipolytic power	Diastatic power	Day*	Lipolytic power		Diastatic power	
					Range in cubic centimeters	Average	Range in hours	Average
1 Dog	4-2	0 35	1 40	1-10	0 38-0 45	0 41	3 00-4 30	3 30
2 Dog	4-2	0 34	1 45	1-17	0 30-0 40	0 34	2 25-4 30	3 15
3 Dog	1	0 50	3 10	1-6	0 40-0 55	0 47	4 30-5 30	5 00
4 Dog	1	0 45	1 05	1-2	0 47-0 60	0 53	4 00-4 10	4 00
5 Dog	1	0 35	2 00	2		0 80		5 00
6 Dog				14		0 57		
7 Dog				7		0 45		
8 Dog	6-1	0 45		2-7	0 45-0 55	0 50		
9 Dog				2-9	0 40-0 55	0 50		
10 Rabbit	11-1	1 00	{	2-5	0 65-0 70	0 68		
				11-25	0 90-0 95	0 95		

\* The figures in these columns give the days before and after the operations, on which the tests were made

## 2 Ligation of the pancreatic ducts and stimulation of the pancreas

Attempts to vary the lipolytic power of the blood by cutting off the external excretion were made in nine dogs. These experiments consisted in (1) tying off the pancreatic ducts, (2) ligating the pancreatic ducts and tissue between the pancreas and the duodenum, (3) extirpating the middle third of the pancreas. In seven of these animals the diastases were also tested and these also show the typical rise in the diastatic curve following obstruction of the pancreatic secretion. The lipase concentration, as will be seen from the following representative protocol, remained normal. Stimulation of the pancreas by feeding also produced little or no effect.

In experiments 11 and 15 to 17, the object was to study the effect of atrophy, for, if the pancreas is the main source of the blood lipase, degeneration of the gland ought to be accompanied by a corresponding decrease in the serolipase. As was mentioned before, only partial atrophy was obtained because of the reestablishment of the external secretion. It was possible, through the kindness of Professor Bensley, to test the blood of a rabbit (pan-

TABLE III

*Detail of Experiment 12 Dog Ligation of pancreatic ducts, March 28, 1911*

DATE	LIPOLYTIC POWER	REMARKS
March 23	0 37	
March 27	0 35	
March 28, 8 a m	0 39	Ligation of ducts 1 00 p m
March 29, 12 m	0 51	Three hours after feeding
March 30, 1 p m	0 47	Three and one-half hours after feeding
March 31, 10 a m	0 40	Three and one-half hours before feeding
March 31, 3 p m	0 41	Three and one-half hours after feeding
April 1, 9 a m	0 40	Three and one half-hours before feeding
April 4, 10 a m	0 36	Three and one-half hours before feeding
April 4, 3 p m	0 41	Four hours after feeding

TABLE IV

*Summary of experiments on obstruction of the external secretion of the pancreas Dogs*

Days

EXPERIMENT	NORMAL				AFTER OPERATION			
	Day	Lipolytic power		Diastatic power	Day	Lipolytic power		Diastatic power hours
		Range	Average	In hours		Range	Average	Range
<i>Ligation of pancreatic ducts</i>								
11*				2 00	7-29	0 30-0 36	0 32	0 15-2 15
12	5-1	0 35-0 37	0 36		1-7	0 36-0 51	0 42	
13	2-1	0 37-0 40	0 39		1-7	0 43-0 54	0 49	
<i>Ligation of pancreatic ducts and tissue adjacent the duodenum</i>								
14	10-1	0 35-0 38	0 37	2 00	1		0 35	
15*				1 50	17-38	0 35-0 41	0 37	0 12-0 45
					42		0 32	
16*				2 00	9-39	0 27-0 36	0 32	0 50-1 45
17*				2 00	6-31	0 27-0 42	0 35	0 12-1 25
<i>Ligation of pancreatic ducts and extirpation of the middle third of the gland</i>								
18	1		0 45	1 50	3-7	0 37-0 57	0 47	0 07-0 07
19	1		0 50	2 00	3		0 45	0 10

\* Considerable atrophy of the

\* Considerable atrophy of the pancreas at autopsy

creatic duct ligated August 27, 1909) the autopsy of which (May 2, 1911) showed complete atrophy of the pancreas except a very small piece, about 3 mm in diameter, which apparently had regenerated from the end of the duct close to the intestine. Several duplicate tests of the serum invariably gave a lipolytic power of from 2.75 to 2.85 cc. Serum of a normal rabbit tested on the following day titrated 2.45 cc. Other normal rabbits were tested for several consecutive days in June, and, while the values varied between the individuals, they remained very constant for each animal.

RABBIT NUMBER	1	2	3	4	5	6	7	8	9	10	11	12
Lipolytic power	1.053	801	051	251	353	052	603	303	001	003	203	40

It is seen that partial atrophy of the pancreas is not followed by any change in the lipolytic power of the serum. And the rabbit, with almost complete atrophy of the gland, certainly showed little or no decrease in the serolipase.

### 3 Ligation of the renal arteries

The results of the ten experiments of ligation of the renal arteries are summarized in Table V. It will be noticed that, in one case of simple ligation of renal arteries (experiment 22), and in both experiments 12 and 13, where the renal arteries were tied off one week after occlusion of the pancreatic ducts, no larger and definite change in the serolipase followed. In all of the other experiments there resulted an immediate increase which gradually fell but usually remained above normal and rose again shortly before death. This curve appears to be independent of and is not materially altered by simultaneous pancreatic lesions, neither does the rise necessarily follow simple renal artery ligation.

### 4 Influence of ether

In order to eliminate the possibility of the anaesthetic being a factor in the renal artery experiments, two dogs were etherized for two hours and blood drawn before, after and every 30 minutes during the anaesthesia. The serum of both dogs showed lipase

TABLE V

*Ligation of renal arteries with and without experimental pancreatic lesions  
Dogs*

EXPERIMENT NO	BEFORE LIGATION			AFTER LIGATION		
	Day	Lipolytic power		Hour	Lipolytic power	
		Range	Average		Range	Average

*Ligation of renal arteries*

20	4-1	0 37-0 45	0 40	2		0 95
				17-27	0 70-0 50	0 60
				43-67	0 60-0 80	0 65
21	3-1	0 45-0 55	0 50	2		0 55
				17		1 70
				21-45	1 20-0 95	1 10
22	7-1	0 40-0 65	0 51	2-52	0 60-0 45	0 45
23	6-1	0 39-0 50	0 42	1		1 60
				6		1 10
				8-53	0 60-0 70	0 65

*Ligation of renal arteries 7 days after ligation of the pancreatic ducts*

12	12-1	0 35-0 51	0 40	2-63	0 40-0 57	0 49
13	9-1	0 37-0 54	0 47	3-69	0 47-0 52	0 48

*Ligation of renal arteries and pancreatic ducts*

24	28-3	0 40-0 56	0 46	3		0 92
				17-21	0 62-0 65	0 65
				43-47	0 94-0 97	0 95
25	22-1	0 32-0 40	0 37	5-8	1 30-1 44	1 37
				11		1 15
				32-56	0 70-0 90	0 77

*Ligation of renal arteries and pancreatectomy*

26	28-1	0 35-0 55	0 45	2-6	0 80-0 92	0 86
				20-29	1 90-2 30	2 10
27	22-1	0 34-0 45	0 40	2-6	0 60-0 80	0 70
				20-29	1 10-1 60	1 20



values between 0.44 and 0.50 cc in all tests while the diastases also remained normal

### 5 Diuresis

Since the concentration of lipase in the serum of dogs was found generally to be higher during the hot weather, a study of the relation of the serolipolytic power to the rate of the kidney and lung excretion suggested itself. The action of increased kidney secretion was tried in a number of dogs. The production of polyuria, by ligation of the renal nerves, or by administration of diuretics of the xanthine group, did not prove entirely satisfactory. Physiological salt solution given by stomach tube is followed by an extensive hypersecretion of urine in the most normal way possible. However, even this method, followed in a series of animals, gave but a slight decrease in the lipase concentration of the serum and this decrease is exactly parallel to the dilution of the blood. A typical experiment of this series is given in Table VI.

TABLE VI  
*Effect of diuresis on serolipase concentration of the dog*

TIME	BLOOD		0.9 PER CENT NaCl SOLUTION PER CC	URINE
	Red cell count	Lipolytic power		
		cc	cc	cc
July 25	8 00 a m	4,600,000	0.45	850
	8 30 a m	4,500,000		
	9 00 a m	4,000,000	0.37	500
	10 00 a m	3,600,000	0.32	570
	11 00 a m	3,700,000	0.33	800
	12 00 m	3,500,000	0.28	
	1 00 p m	4,000,000	0.36	
	2 30 p m	4,000,000	0.40	
	7 00 p m	4,600,000	0.37	
July 26 8 00 a m	4,700,000	0.41		300
July 27 8 00 a m	not taken	0.44		not taken

### 6 Hyper- and hypo-thyroidism

Investigation of a large number of normal rabbits showed a possible variation between individuals of a concentration of lipase of the serum from 0.75 to 4.00 cc. A rabbit which had died from

forced thyroid feeding showed a lipase value of 0.55 cc. Five others killed after two weeks of forced thyroid feeding gave the following values: 0.95 cc, 0.70 cc, 1.65 cc, 1.90 cc, and 1.75 cc respectively. It is evident that "hyperthyroidism," as produced by forced thyroid feeding, does not vary the serolipolytic power at least beyond the normal variation of this species. Juschtschenko<sup>1</sup> claims that in the dog hyperthyroidism always increases the lipase concentration of the blood while hypothyroidism decreases it. In this laboratory thyroidectomy in three dogs gave lipolytic figures of 0.35 cc, 0.39 cc and 0.50 cc respectively, with blood drawn from 5 to 14 days after operation. These values are well within the limits of normal individual variation and do not confirm the results of the above author. Indeed, it seems that the data in the author's own paper hardly justify the conclusions drawn. In view of the range of variations of the serolipase in normal dogs the small changes, which he obtained, might easily be explained on the basis of individual variation.

#### 7 *Role of the leucocytes*

The relation of the leucocytes to the production of the blood lipase was also determined in one dog. By injecting aleuronat into one pleural cavity inflammation ensues and an exudate rich in leucocytes fills both cavities. The exudate was tested twenty-four hours after the injection. The lipolytic power of this exudate was 0.25 cc as against 0.20 cc of a boiled control, while the serum from the same animal showed no change from the normal concentration of 0.35 cc.

#### IV DISCUSSION OF RESULTS

As will be seen from Table II in the series of experiments on pancreatectomy in dogs, the diastases of the serum show an abrupt decrease in concentration with little or no return towards the normal, while the lipase content presents only slight changes that are easily accounted for by individual and daily variation. If the pancreas, either directly through simple absorption, or, indirectly by influencing other organs through an internal secretion,

<sup>1</sup> Juschtschenko *Biochem Zeitschr*, xxv, p 49, 1910

is an important factor in the source of blood lipase, one would be justified in supposing that on removal of the gland there should result a decrease in the lipolytic power of the serum. It is possible that there is a decreased destruction of the ferment after pancreatectomy, as the narrow range of its concentration points to a delicate mechanism governing the rate of production and destruction or elimination. The temporary fall obtained in the rabbit (experiment 10) would seem to be due to a decreased production in the other tissues of the body owing to the general depression which results from the severity of the operation rather than to the mere absence of the gland itself.

In the ligation of the pancreatic ducts there is likewise a striking contrast between the lipase and the diastases. As stated before the diastases are greatly increased within twenty-four hours. This rise is probably due to absorption from the pancreas. The gland becomes edematous and absorption results from back pressure. But the lipase curve presents no variations. There is no doubt that, after ligation, pancreatic lipase is absorbed by the blood, for Hewlett<sup>1</sup> found an increase of the lipase in the urine under the same experimental conditions. Apparently the excess of lipase is excreted as fast as it is absorbed. The negative results obtained in the stimulation of the pancreas may also be explained in the same way. And the fact that partial atrophy of the gland in dogs and practically complete atrophy in one rabbit after ligation is accompanied by no deviation in the lipase concentration of the serum, points to some sort of a compensatory mechanism.

Shutting off lipase elimination through the kidney, by tying the renal arteries, in two dogs with ligation of pancreatic ducts one week previous, produced no change. This may be explained in three ways: (1) the pancreatic lipase was no longer absorbed by the blood, (2) there was an increased destruction of the ferment within the body, (3) the absorbed enzyme was stored away in certain tissues.

Autopsy in both dogs showed the pancreas in an edematous condition which fact seems to argue against the first explanation. A comparison, of the experiments of simple ligation of renal arteries, and of pancreatic ducts and renal arteries, shows the lipase

<sup>1</sup> Hewlett *Loc cit*

curves to be the same and indicates that, in the latter, the absorption of the pancreatic lipase is not significant. If, according to our second hypothesis above, there was an increased destruction of the lipase after its absorption from the pancreas, this same theory fails to account for the enormous increase of serolipase that is alike in both sets of experiments. The results of ligation of renal arteries with pancreatectomy also support this conclusion. The rise is not due to the ether anaesthesia, for the ether controls are negative. What seems probable, then, is that neither of the first two explanations above can account for the results, but, that this sudden increase in serolipase concentration is due to the hyperactivity of all the tissues in general as a result of intense stimulation through the accumulation of toxic metabolic products in the blood. The gradual decrease, following the maximal rise, would represent the lessened cellular activity. The fact, that in three instances no change followed renal artery ligation, shows that accumulation of toxic substances in the blood need not necessarily increase this activity of the tissues.

All the results point to one conclusion—that the concentration of the lipase of the serum can not be markedly altered by experimental variations in the activity of single organs but only by variations in the activities of the tissues in general. This means, then, that there is a certain equilibrium between the tissues and the blood, so far as the lipase is concerned, and the disturbance of this balance on either side is followed by a tendency towards reestablishment. But it is evident that the amount of tissue lipase is so vastly greater than the serolipase, that the alteration of any single organ will not appreciably affect the concentration of the lipase in the serum, since the remaining tissues can restore the equilibrium without proportionately changing their lipase concentration. Indeed, a regulator, so sensitive as is the lipase mechanism, can hardly be conceived of maintaining such an extreme variation of levels of serolipase concentration, as exists between species and even between individuals of the same species, unless tissue activity in general is one of the controlling factors. Thus, the bird, extremely active and demanding a high muscular activity, has a high serolipase concentration, while the goat and the sheep, much less active and requiring lower tissue activity, show only a trace in the lipolytic power of the serum.

It seems probable on the basis of the above hypothesis that, by varying the lipase absorption from a single organ such as occurs in the experiments on the pancreas, the lipase concentration of the serum should not be materially affected and that, on the other hand, renal artery ligations and operative procedures may alter the concentration because the activity of the body cells in general are affected.

Attempts to lower the lipolytic power of the serum by increased elimination in the urine proved negative. It was found that diuresis, induced by ingestion of physiological salt solution *per os*, gave a small but constant drop paralleling the curve of blood dilution. The lipase excretion, if any at all, was too small to be determined. It is known that lipase in solution has a strong tendency to be carried down with precipitating colloids, and it is probable this lipase is loosely combined with certain proteins and owes its stability to such combination. The lipase, normally present in the blood, may be hitched on to the blood proteins and hence not eliminated in normal urine secretion or in diuresis. On the other hand, following pancreatic lesions, an excess of lipase may be absorbed by the blood and excreted in the urine before it has become attached to larger colloidal molecules.

Various authors have shown differences in the activity of the lipase of serum and that of tissue extracts, especially as regards velocity of reaction, leading to the conclusion that the lipases differ from one another. It is possible that no two lipase extracts from different tissues act precisely alike, and, in view of the work reported in this paper, a more likely explanation of these differences is that they are not different lipases but the same lipase in different combination with the tissue colloids.

Experiments on the lipolytic power of the serum of dogs in hypothyroidism gave results within the range of individual variation. This is to be expected if, as in the case of the pancreas, serolipase is the product of cellular activity in general and not of any particular organ. The experiment on leucocytosis also points to a similar conclusion. While a serous exudate, produced by aleuronat injection into the pleural cavity, gave some lipase activity, it was less than that of the serum of the dog and may have been the results of filtration or secretion.

It has been suggested that the lipase in the blood is present in an inactive state, and, in experimental conditions, may be absorbed by the blood as such, in which case, the negative results in changes of the serolipase following pancreatic and thyroid lesions might be explained on this basis. But the blood serum, tested with the addition of bile salts as activators in both normal and abnormal conditions produced in the course of the experiments, gave no increased action on ethylbutyrate.

The increase in the lipolytic power of the serum on very hot days is probably due to concentration of the lipase because of diminished urine secretion and increased elimination of water from the lungs.

#### V CONCLUSIONS

1 Extirpation of the pancreas in dogs does not alter the concentration of the lipase in the serum beyond the range of individual and daily variations. Rabbits, after pancreatectomy, may show a temporary fall which soon returns to normal.

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3 Partial atrophy of the pancreas in dogs and almost complete atrophy of the gland in the rabbit produces no change in the serolipase, nor is there any change in the lipase concentration parallel with the degree of functional activity of the pancreas with or without the ligation of the pancreatic ducts.

4 (a) On mere ligation of renal arteries in dogs, normal or following previous ligation of pancreatic ducts, or, (b) on ligation of renal arteries simultaneously with ligation of the pancreatic ducts or with extirpation of the pancreas, the lipase concentration of the blood may or may not change. In case it does, there is a rather abrupt increase followed by a gradual fall, which, however, still remains above the normal and may rise again shortly before death. The curve is independent of the accompanying pancreatic lesions and seems to result only from the occlusion of the renal arteries.

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in a slight decrease of serolipase concentration which is due entirely to blood dilution

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10 The above results indicate that the pancreas and the thyroid are not the main but only partial source of the blood lipase and only in so far as they play their part in the activity of the tissues in general. Serolipase variations are produced by a general stimulation or depression of the body cells, while the small changes, which experimental lesions of these glands may produce are compensated for through the delicate mechanism governing the concentration of the serolipase

I wish to express my thanks to Professor A. J. Carlson for his assistance and many valuable criticisms and suggestions given during the progress of this work

# SOME NEW COMPOUNDS OF THE CHOLINE TYPE

(Preliminary Paper)

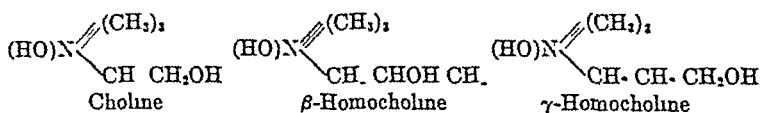
By G. A. MENGE

(From the Hygienic Laboratory, U. S. Public Health and Marine Hospital Service)

(Received for publication, October 30, 1911)

So far as the writer is aware the synthetic development of compounds of the choline type has not extended beyond the preparation of choline, the so-called homocholines ( $\beta$  and  $\gamma$ ), some esters of these and certain other derivatives, reference to practically all of which is made in the work of Hunt and Taveau on the effects of such compounds on the blood-pressure<sup>1</sup>

The relation of the homocholines, referred to above, to choline itself is shown in the following formulas



the nomenclature being based upon the position of the hydroxyl in the carbon chain with reference to the nitrogen

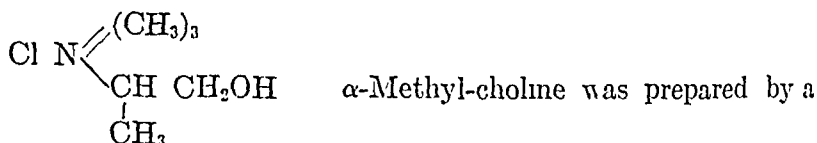
The physiological activity of these compounds and their derivatives as determined by the work reported in Bulletin 73, indicates in a general way that the greatest activity and least toxicity is realized in those compounds which contain the trimethylamine nucleus and in which the alcoholic hydroxyl is not further removed from the nitrogen than the  $\beta$ -position. That is, the substitution of the  $\alpha$ - and  $\beta$ -hydrogen of the carbon chain by alkyl residues, etc., rather than the direct extension of the chain, was indicated as the most promising development from the standpoint of physiological activity.

<sup>1</sup> Hunt, R. and Taveau, R. de M. *Hygienic Laboratory Bull. No. 73*, U. S. Public Health and Marine Hospital Service



Probably the simplest method of nomenclature for such a class of compounds would be to consider them as substituted cholines, with the further designation of  $\alpha$ - or  $\beta$ - according as the substituted group is linked to the first or second carbon. From this point of view the  $\beta$ -homocholine given above becomes  $\beta$ -methylcholine.

The first new compound obtained in line with the suggested development was  $\alpha$ -methylcholine in the form of its chloride<sup>1</sup>



somewhat indirect method. Allyl chloride was converted, by the method of Oppenheim,<sup>2</sup> into the chlorhydrine,  $\text{CH}_2\text{CHOHCH}_2\text{Cl}$ . The chlorine of this compound was replaced by an acetate group according to the method of Henry<sup>3</sup> yielding the acetate,  $\text{CH}_3\text{COOCH}_2\text{CHOHCH}_3$ , which in turn was treated with hydrochloric acid<sup>4</sup> yielding the chlor-acetate,  $\text{CH}_3\text{COOCH}_2\text{CHClCH}_3$ , and this compound on saponification with dry hydrochloric acid in absolute methyl alcohol<sup>5</sup> yielded the desired chlorhydrine,  $\text{CH}_3\text{CHClCH}_2\text{OH}$ .

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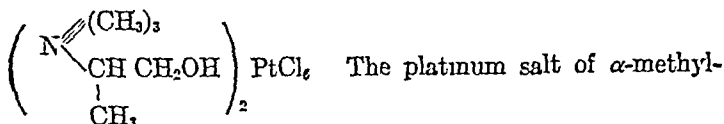
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bulb, the alcohol distilled off *in vacuo*, and the residue washed several times with methyl alcohol (each wash portion being distilled off *in vacuo*) to remove all excess of trimethylamine. The final product thus obtained consisted of a yellowish, very viscous oil, from which a white crystalline solid,  $\alpha$ -methyl-choline, gradually separated on cooling. This solid proved to be extremely hygroscopic. It was identified and analyzed by means of the platinum salt

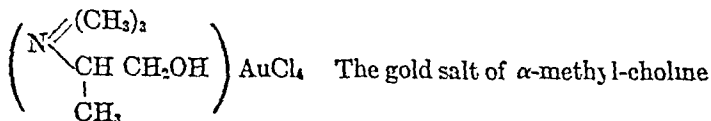


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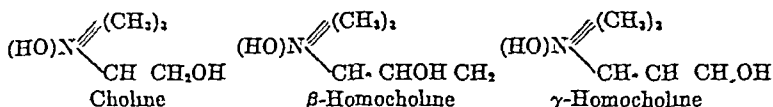
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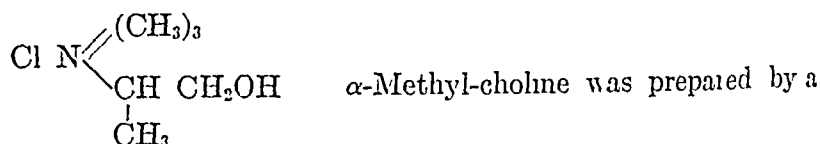
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The physiological activity of these compounds and their derivatives as determined by the work reported in Bulletin 73, indicates in a general way that the greatest activity and least toxicity is realized in those compounds which contain the trimethylamine nucleus and in which the alcoholic hydroxyl is not further removed from the nitrogen than the  $\beta$ -position. That is, the substitution of the  $\alpha$ - and  $\beta$ -hydrogen of the carbon chain by alkyl residues, etc., rather than the direct extension of the chain, was indicated as the most promising development from the standpoint of physiological activity

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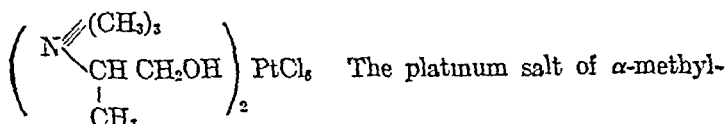
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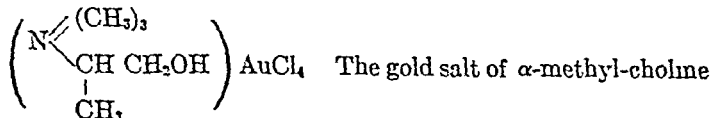


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III	0.1512 gram salt gave 0.0460 gram Pt

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Pt	30.26 per cent		30.33 per cent	30.36 per cent		30.42 per cent	



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was prepared by dissolving the  $\alpha$ -methyl-choline in a little water and precipitating with an aqueous solution of chlorauric acid. The resulting pale yellow precipitate was at first more or less oily but became definitely crystalline on rubbing. On heating it sinters gradually above  $180^\circ$  and slowly melts at  $198^\circ$  to  $199.5^\circ$ .

Analysis for gold resulted as follows

0.1917 gram salt gave 0.0827 gram Au

	Calculated for $C_6H_{15}ONCl_4$ Au	Found
Au	43.13 per cent	43.14 per cent

In general the gold salts do not lend themselves as readily to convenient manipulation as do the platinum salts.

The acetyl, phenyl-acetyl, valeryl, benzoyl, mono-brom-iso-capronyl, and palmityl derivatives of  $\alpha$ -methyl-choline, together with their platinum and gold salts, have also been prepared but their description will be reserved for a later paper.

The physiological activity of  $\alpha$ -methyl-choline and its acyl derivatives<sup>1</sup>—more especially the acetyl derivative—has justified the promise offered in the development of this class of compounds and stimulated effort to further extend it.

A preliminary detail of primary importance to such development is the preparation of chlorhydrines of the desired structure. Several means to that end were under consideration—among them, the possibility of reducing a cyanhydrine to an amino-alcohol, replacing hydroxyl with chlorine, and substituting hydroxyl for the amino group by means of the diazo reaction, seems worthy of mention because of the great variety of chlorhydrines its successful application would make available. Two methods for reducing the cyanhydrines were at once suggested: (1) by the Mendius reaction<sup>2</sup> for the reduction of nitriles—disregarded because of low yields—and (2), the Landenberg method<sup>3</sup> for the reduction of nitriles. The latter was applied in two attempts to reduce cyanhydrines but without success—doubtless due to the tendency

<sup>1</sup> All pharmacological investigation to which these compounds have been applied has been made by Dr. Reid Hunt, Professor of Pharmacology in this laboratory.

<sup>2</sup> Mendius, *Ann. der. Chem.*, cxi, pp. 129–153.

<sup>3</sup> Landenberg, *Ber. d. deutsch. chem. Gesellsch.*, xviii, p. 2957, 1885.

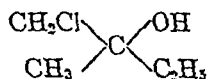
of these compounds, in an alkaline medium, to revert to the corresponding aldehyde or ketone by splitting of hydrocyanic acid. The writer is hopeful, however, that he may be able to devise some modification of the Ladenberg method to offset this tendency.

A second method considered for the preparation of the type of chlorhydrines desired, consists in the well-known condensation of hypochlorous acid with the olefines. The structure of the chlorhydrines obtained by this method, however, seems to be a matter of much controversy among different able investigators.<sup>1</sup> From a pharmacological point of view it is important that in these choline compounds there should be the least possible doubt regarding structure and therefore this method for the preparation of the chlorhydrines seemed undesirable if a more certain one were available.

Various investigators have shown that Grignard's reaction for the synthesis of tertiary alcohols is readily adaptable for chlorhydrines of similar type and concerning the structure of which there seems to be no doubt.

Using monochloroacetone<sup>2</sup> with different alkyl-magnesium-halides the writer has successfully applied the method to the preparation of two such chlorhydrines,—the chlorhydrine of

dimethyl-glycol,  $\begin{array}{c} \text{CH}_2\text{Cl} \\ \diagup \\ \text{C} \\ \diagdown \\ \text{CH}_3 \end{array} \begin{array}{c} \diagup \\ \text{OH} \\ \diagdown \\ \text{CH}_2 \end{array}$ , and of ethyl-methyl-glycol,<sup>3</sup>



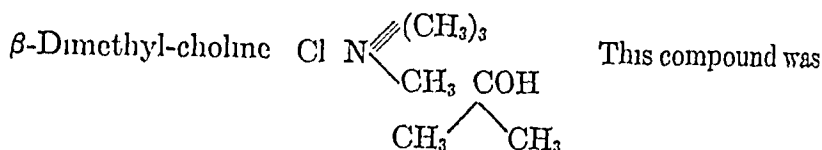
Obviously these compounds condensed with trimethylamine would yield  $\beta$ -disubstituted cholines—a new class of compounds. It is intended, however, to attempt the transposition of the chlorine and hydroxyl in chlorhydrines of this type, thus leading to the corresponding  $\alpha$ -disubstituted cholines.

A brief description of the two new choline compounds derived from the above-mentioned chlorhydrines follows.

<sup>1</sup> Butlerow, Henry, Markownikoff, Michael, Krassusky, Tiffeneau and others.

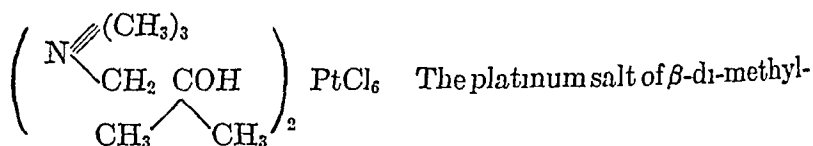
<sup>2</sup> Prepared according to Fritsch, *Liebig's Annalen*, cclxxxv, pp 310-315.

<sup>3</sup> Both compounds have previously been prepared by Tiffeneau *Compt rend acad d sci*, cxxxv, p 775, and other investigators.



prepared by mixing the chlorhydrine of dimethylglycol with a calculated quantity (in slight excess of theory) of a 33 per cent solution of trimethylamine in absolute alcohol and heating the mixture in a sealed tube to  $100^\circ$  for about four hours. The reaction product, on cooling, was poured into a distilling bulb and the alcohol and excess of trimethylamine evaporated off under diminished pressure, aided by gentle heating over a water-bath, leaving a slightly colored, very viscous, oily residue. This residue was washed several times with methyl alcohol, each wash portion being evaporated off as above described.

The product thus obtained was partly an oil and partly a beautifully crystalline white solid. By subjecting this mixture to long continued diminished pressure the whole mass became solid though slightly contaminated by coloring matter. Apparently a very little alcohol or a trace of unaltered chlorhydrine or a by-product contamination, is sufficient to maintain a considerable portion of the product in the form of an oil. The solid finally obtained is very soluble in alcohol. The addition of dry ether to a concentrated solution of the product in absolute alcohol caused abundant precipitation of a perfectly white solid, which is very hygroscopic and, based upon analysis of the platinum salt, proved to be pure  $\beta$ -dimethyl-choline.

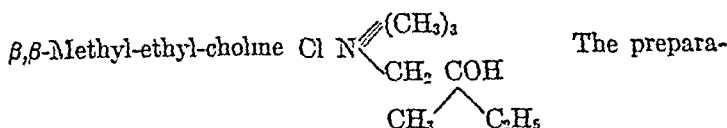


choline was prepared by dissolving the  $\beta$ -methyl-choline in absolute alcohol and precipitating with alcoholic solution of chlor-platmic acids. The yellow precipitate obtained is practically insoluble in absolute alcohol, fairly soluble in cold water, and readily soluble in hot water from which it crystallizes in short individual prisms or foliated clusters. Heated in a capillary tube the salt gradually darkens in color, begins to blacken at about  $240^\circ$  and decomposes with vigorous effervescence at  $245^\circ$ .

Analyses for platinum gave the following duplicate results

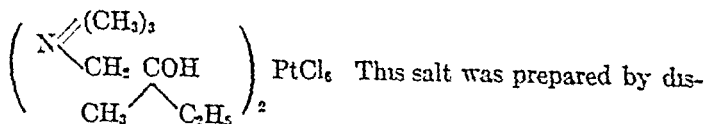
- I 0.1080 gram salt gave 0.0313 gram Pt  
 II 0.1035 gram salt gave 0.0301 gram Pt

	Calculated for $C_{11}H_{20}O_2N_2Cl_2Pt$	I Found	II Found
Pt	28.997 per cent	28.98 per cent	29.08 per cent



tion of this compound was accomplished in a manner analogous to that of the  $\beta$ -dimethyl-choline, except that the reaction mixture was heated in a tube furnace to about  $150^\circ$  to  $160^\circ$  for about three hours. Even so the condensation was apparently not complete and the reaction was productive of much more "schmiere" than in the previous preparation, though this feature may have been due in some measure to impurity in the chlorhydrine used. It is also probably true that the preparation of the higher homologues and other more complex derivatives of this type of choline compounds will offer progressively increasing difficulty in obtaining smooth reaction and clean products. However, the point of immediate importance in this preparation was to determine the presence or absence of the desired compound, leaving the problem of its separation in pure form for subsequent investigation.

The crude reaction product in this instance was treated as in the preparation of the  $\beta$ -dimethyl-choline, yielding a residue of very viscous oil mixed with a large proportion of crystalline solid which appeared nearly white in a thin layer though the mass was much blackened by "schmiere". The presence of the  $\beta, \beta$ -methyl-ethyl-choline, in very considerable proportion, was determined by means of the platinum salt.



solving a portion of the crude product, described above, in absolute alcohol and adding an alcoholic solution of chlorplatinic acid. A

voluminous, very finely divided, yellow precipitate was obtained, it was filtered with difficulty, and washed with absolute alcohol. The salt is practically insoluble in absolute alcohol, quite soluble in cold water and very soluble in hot water. It can be recrystallized from hot water but much more easily from a hot mixture of alcohol and water. A portion of the crude salt was treated with a quantity of a mixture of alcohol and water (about 1 l) insufficient to dissolve all the salt. The mixture was heated with stirring on a boiling water-bath and filtered hot. The crystals separating from the filtrate were filtered off, washed and dried in an air bath at 100° to 110° C. On heating in a capillary tube the salt sinters at about 240° and melts with decomposition at 242° to 243°.

Duplicate analyses for platinum resulted as follows

	I	0.1429 gram salt gave 0.0396 gram Pt
	II	0.1471 gram salt gave 0.0410 gram Pt
	Calculated for $C_{10}H_{16}O \cdot N_3Cl_4Pt$	
	Found	
	I	II
Pt	27.836 per cent	27.87 per cent

Platinum analyses on the crude platinum salt gave results about 2 per cent high, while analyses on the undissolved residue in recrystallizing the crude salt gave results over 3 per cent high, suggesting the presence of unaltered trimethylamine in the crude reaction product and offering corroborative evidence of incomplete condensation of the trimethylamine and chlorhydrine.

It is intended to further pursue the development of this type of choline compounds and their various derivatives, and to determine their physiological action, in this laboratory as rapidly as the demands of other work permit.

# INTERRELATION OF THE AMMONIA AND CARBON DIOXIDE CONTENT OF THE BLOOD

BY RALPH HOPKINS AND W. DENIS

*(From the Physiological Laboratory of the Tulane Medical School)*

(Received for publication, October 30, 1911)

The problems underlying the present research are (1) To what extent does carbon dioxide exert a beneficial action in binding ammonia set free by protein metabolism (2) Does a lack of carbon dioxide lead to an accumulation of ammonia and if so might this fact account for some of the respiratory changes usually attributed to lack of carbon dioxide alone (3) Is it possible by means of respiration experiments to draw conclusions as to the relative ease of the transformation of ammonium carbonate, ammonium lactate, etc., into urea by the liver Thus an increase in the amount of carbon dioxide in the blood would lead by mass action to an increased amount of ammonium carbonate and the latter by conversion into urea would no longer act as an ammonia salt and therefore the total ammonia content of the blood should fall On the other hand if a diminution in the normal carbon dioxide content of the blood is produced artificially we ought to get an increase in the total ammonia content provided that the free ammonia is combined with some acid other than carbon dioxide and is thereby less easily converted into urea

## EXPERIMENTAL METHODS

With a few exceptions (see later) our experiments were performed on adult dogs which had been fed on meat twenty hours previously As anaesthetic, ether was used in a few cases but as a rule chloretone in dilute alcoholic solution was administered either by mouth or by rectum

As the ammonia content varies greatly in different individuals we took, for comparison, in every experiment a sample of blood

under conditions as nearly normal as we could make them. Unless otherwise stated all samples of blood were drawn from the carotid artery directly into a 25 cc volumetric flask containing 5 cc of a 1 per cent sodium fluoride solution. This procedure prevented clotting entirely. As a rule only two samples (that is 40 cc in all) were taken from any one animal to avoid as much as possible complications which might arise from a too rapid or too great depletion of the vascular system. All ammonia determinations were made by the Folin method, using centinormal acid and alkali.

### EXPERIMENTAL RESULTS

*Partial asphyxiation* In the first series of experiments the carbon dioxide content of the blood was increased by preventing the ready elimination of this gas. The animal was made to breathe through a tracheal canula which was connected to a rubber tube about two feet long and half an inch in diameter. This tube passed into a beaker, of one and a half liters capacity, floated upside down in another vessel of water. The beaker was allowed to rise and fall with expiration and inspiration, with this apparatus asphyxiation could be produced in from six to fifteen minutes. In every case the animal was allowed to recover for from fifteen to thirty minutes from the effects of the insertion of the tracheal cannula and the exposure of the carotid artery before any blood was taken. In two cases the normal blood was drawn just before respiration was interfered with and the asphyxiated blood as soon as the first symptoms of asphyxial convulsions were seen. In the other two cases the order of the taking of samples was reversed, that is partial asphyxia was produced, a sample taken, then the animal allowed to recover for about thirty minutes before taking the second sample which was regarded as normal.

As will be seen by the table on the following page an average diminution in ammonia of about 50 per cent was obtained, which result we felt justified in attributing either to an excess of carbonic acid or to a lack of oxygen or to both.

*Effect of artificial respiration* In our earlier experiments we sought to diminish the carbon dioxide content of the blood by means of artificial respiration carried on as follows. A current of air was introduced through a rubber catheter which was passed

through a T-shaped tracheal cannula to the bifurcation of the bronchi. The catheter was of sufficiently small size to allow the air which had been introduced to readily find its way out through the space between the catheter and the inside surface of the cannula and then through the unobstructed side arm of the cannula. The other arm, through which the catheter was introduced, was closed by a cork through which the catheter passed to prevent an escape of the return air by this channel. The inflation of the lungs was accomplished by closing the unobstructed side arm of the cannula, and the inflation could be regulated easily as to frequency and extent by closing the opening with a finger and by varying the

DOG	DATE OF EXPERIMENT	SEX	WEIGHT	NH <sub>3</sub> IN 100 CC NORMAL BLOOD	NH <sub>3</sub> IN 100 CC ASPHYX- IAL BLOOD	REMARKS
				grams	mg	
1	December 27, '10	♀		2.6	1.3	Ether anaesthesia
2	January 4, '11	♂	10320	1.8	0.9	Ether anaesthesia
3	January 5, '11	♂	8900	1.9	0.9	Anaesthetized with 2.25 grams chloretone ad- ministered by mouth
4	January 6, '11	♀	9900	1.8	0.88	Anaesthetized with chloretone

amount of pressure in the reservoir which supplied the current of air. The pressure used was equal to about 2 feet of water pressure. In the first experiments with this apparatus the results were contrary to our expectations, as the amount of ammonia was considerably diminished. On taking, however, a record of the blood pressure our method was found to produce a great fall in it, as the artificial distension of the lungs interfered mechanically with the entrance of blood into the thorax, for it must be remembered that animals during artificial respiration carried out as described above make no effort to breathe, and that therefore the distension of the thorax is brought about by positive pressure within the lungs. It was surprising to find how great a fall in blood pressure could be produced by a comparatively moderate degree of distension of the lungs.



# 410 Ammonia and Carbon Dioxide of the Blood

DOG	DATE OF EXPERIMENT	SEX	NH <sub>3</sub> IN 100 CC NORMAL BLOOD	NH <sub>3</sub> IN 100 CC BLOOD, ART RESP, LOW D P	REMARKS
			mg	mg	
5	January 7, '11	♂	1 9	0 9	2 0 grams chloretone by rectum
6	January 12, '11	♀	1 2	0 6	2 0 grams chloretone by rectum
12	January 26, '11	♂	1 5	1 0	2 0 grams chloretone by rectum

The decrease in ammonia shown in the results given in the above table may, we think, be attributed to a decrease in metabolism during the period of low blood pressure

By taking care not to lower the blood pressure by the artificial respiration our surmise as to the cause of the diminished ammonia found in the first experiments with artificial respiration was found to be correct. Where the blood pressure was not interfered with, we found in four dogs an average increase in the ammonia content of the blood of about 30 per cent

From the results given in this table we feel justified in believing

DOG	DATE OF EXPERIMENT	WEIGHT	SEX	NH <sub>3</sub> IN 100 CC NOR- MAL BLOOD	NH <sub>3</sub> IN 100 CC BLOOD AFTER ART RESP	REMARKS
		grams		mg	mg	
7	January 16, '11	7500	♀	1 3	1 8	2 0 grams chloretone by rectum Artificial respiration for twenty minutes
8	January 17, '11		♀	0 9	1 5	2 0 grams chloretone by mouth Artificial respiration for thirty minutes
9	January 19, '11		♀	1 0	1 3	2 0 grams chloretone by mouth, 10 gram by rectum Artificial respiration thirty minutes

that either diminution of carbon dioxide or excess of oxygen or both are responsible for the increased ammonia content of the blood

*Effect of administering carbon dioxide and oxygen* In the next series of experiments we investigated the effect of increasing the carbon dioxide content of the blood without diminishing the oxygen For this purpose the animals were made to breathe an atmosphere consisting of 90 per cent of oxygen and 10 per cent of carbon dioxide The same method of administration was followed as in the artificial respiration experiments except that the exit for the return gases from the lungs was not alternately opened and closed to inflate the lungs but remained open all the time, the natural inspiratory and expiratory movements of the animal not being interfered with

The current of the mixture of gases flowing through the catheter and out through the unobstructed arm of the canula was further carried through a rubber tube 6 feet long to prevent any entrance of air during inspiration through this exit The gases escaping from the free end of the rubber tube were found always to be sufficiently rich in oxygen to cause a smouldering match to burst into flame

DOG	DATE OF EXPERIMENT	SEX	WEIGHT	NH <sub>3</sub> IN 100 CC NOR MAL BLOOD	NH <sub>3</sub> IN 100 CC BLOOD AFTER ART RESP	REMARKS
			grams	mg	mg	
13	January 27, '11	♂		1.4	1.6	2 grams chloretone by rectum Artificial respiration for thirty minutes
14	January 28, '11		8320	1.4	1.7	20 grams chloretone by rectum Artificial respiration for forty-five minutes
16	January 29, '11	♂	5650	1.6	1.8	20 grams chloretone by mouth Twenty minutes artificial respiration
18	March 3, '11	♀	8650	0.6	1.0	20 grams chloretone by rectum Twenty minutes artificial respiration

## 412 Ammonia and Carbon Dioxide of the Blood

We believed the normal blood to be so nearly saturated with oxygen that the excess of this gas in the above mixture would not materially affect the production of ammonia, our experimental results, however, proved this surmise to be incorrect, for instead of obtaining a decrease in the ammonia content of the blood due to the accumulation of carbon dioxide in this fluid we obtained a slight increase. It was noted that the blood when taken for analysis was much brighter than normal, and this fact together with the increased content of ammonia found in the above experiments led us to believe that the inhalation of an atmosphere containing an excess of oxygen produces, at least in anaesthetized dogs, an increase in the ammonia of the blood. With a view of testing the correctness of this surmise several dogs were made to inhale pure oxygen, which procedure was found to produce a marked increase in the ammonia of the blood.

DOG	DATE OF EXPERIMENT	SEX	WEIGHT	NH <sub>3</sub> IN 100 CC "NORMAL" BLOOD	NH <sub>3</sub> IN 100 CC BLOOD AFTER ART RESP	REMARKS
			grams	mg	mg	
17	February 27, '11	♂		2 0	2 5	2 0 grams chloretone by rectum Oxygen administered for thirty minutes
19	March 10, '11		5900	1 0	2 2	2 0 grams chloretone by rectum Oxygen administered for twenty minutes
20	March 17, '11		5800	1 6	2 0	2 0 grams chloretone by rectum Oxygen for twenty-minutes
21	March 17, '11	♀	4800	1 3	2 0	2 0 grams chloretone Oxygen for forty minutes

From a comparison of the average percentages of increase in ammonia obtained in the experiments made with pure oxygen and in those made with a mixture of oxygen and carbon dioxide it would seem that carbon dioxide prevents to a certain extent the accumulation of ammonia, in the series in which oxygen alone was

used there was an average increase in ammonia of 55 per cent, in the series in which a mixture of oxygen and carbon dioxide was used the average increase in ammonia was 28 per cent

The number of experiments made was, however, too small to permit of any final conclusions regarding this theory, and it is also possible that the 10 per cent carbon dioxide added to the oxygen may in some way have prevented as complete an oxidation in the tissues as occurred when the oxygen was administered pure, and that therefore less ammonia was produced

*Effect of curare on the ammonia content of the blood* In the next series of experiments curare was used for we hoped in this way to obtain a reduction in the carbon dioxide formation, with a subsequent increase in the ammonia content of the blood because the curare by rendering the muscular tissue inactive would eliminate the chief source of carbon dioxide

Curare was administered to some of the animals subcutaneously and to others intravenously

An effort was made to take the second sample of blood just as respiration ceased and while the heart was still beating, in all but two dogs this was successfully accomplished, in the case of the two animals in which the heart stopped beating before the amount of blood necessary for the ammonia determination could be drawn from the carotid artery the heart was quickly opened and the flask filled directly from the left ventricle

DOG	DATE OF EXPERIMENT	MILLIGRAMS $\text{NH}_3$ IN 100 CC VORMAL BLOOD	MILLIGRAMS $\text{NH}_3$ IN 100 CC BLOOD AFTER CURARE ASPHYXIA- TION
24	March 31, '11	1 8	2 3
27	April 12, '11	1 9	2 3
28	April 21, '11	3 2	3 4
31	April 26, '11	2 3	2 8

The series of experiments given above shows a small average increase, compared to the series of experiments in which asphyxiation was produced by causing the animal to breathe into a closed vessel we find that asphyxiation in one case has caused a marked diminution in the ammonia content of the blood and in the other case a slight increase

If we remember that curare on account of its paralyzing effect on the muscles is likely to diminish the formation of carbon dioxide, the explanation suggests itself that during the asphyxiation produced by curare poisoning, the ammonia of the blood is less rapidly transformed into urea on account of the diminished supply of carbon dioxide, while as stated above in asphyxiation brought about by breathing into a closed vessel, the accumulation of carbon dioxide favors urea formation.

Further, as oxidation always means increased ammonia formation (see table, p. 412) it follows that in the case of curare poisoning, notwithstanding the diminished ammonia production, there is not sufficient carbon dioxide liberated to bind the diminished output of ammonia.

*Asphyxiation experiments on dogs in full digestion* Some further experiments were made on dogs fed six hours previously on liver, and which, therefore, were in full digestion. The object in view was to determine whether the ammonia liberated in the alimentary canal and during absorption as well as in the liver itself would alter the result we had obtained in fasting dogs. Though this work is not yet completed we wish to state that so far as the results of partial asphyxiation are concerned we have obtained in fed dogs results the very opposite of those we found in fasting animals: that is, we get a rise in the ammonia content of the blood instead of a fall during asphyxiation. The explanation is suggested that during protein digestion a larger amount of ammonia is formed and therefore it would take a larger amount of carbon dioxide to form ammonium carbonate and asphyxiation, giving larger amounts of carbon dioxide, ought to facilitate the formation of urea and thereby lessen the amount of ammonia. The opposite result which we obtained might be due either to a time factor, which means that the liver working to its utmost is not able to cope all at once with the large amounts of ammonium carbonate, or we may assume that because of asphyxiation the liver activity is interfered with and that, therefore, the ammonium carbonate is not as readily converted into urea as would be the case if the liver were fully supplied with oxygen. That the second explanation is the more likely one seems to be proved by the fact that the average amount of ammonia obtained from the normal carotid blood of fed animals is not richer in ammonia than is similar blood

obtained from starving dogs This question is being investigated by us and we hope to be able to give our results in a future communication

#### SUMMARY

1 Asphyxiation of unfed animals by increasing the carbon dioxide content of the blood causes a decrease in the ammonia content of this fluid

2 Artificial respiration of unfed dogs causing a decrease in the carbon dioxide content of the blood causes an increase in ammonia

3 Inhalation of oxygen by unfed dogs increases the ammonia content of the blood

4 Curare slightly increases the ammonium content in unfed dogs

5 Low blood pressure by decreasing oxidation decreases the ammonia content of the blood in unfed dogs

6 Asphyxiation of dogs in full digestion gives an increase of ammonia instead of a diminution as in starving dogs

In conclusion the authors wish to express their appreciation of the numerous suggestions and of the helpful criticism which they have received from Professor Gustav Mann during the entire course of this work



## FASTING STUDIES V (STUDIES ON WATER DRINKING XI)

### THE INFLUENCE OF AN EXCESSIVE WATER INGESTION ON A DOG AFTER A PROLONGED FAST

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(Received for publication, October 11, 1911 )

In the very extensive literature of fasting, in so far as we are aware, there is nothing recorded as to the distribution of the urinary nitrogen of the fasting organism under the influence of a voluminous water intake. Of the experiments on record regarding the influence of water ingestion upon nitrogen excretion those by Voit,<sup>1</sup> Forster,<sup>2</sup> Rubner<sup>3</sup> and Fraenkel<sup>4</sup> are the most important. The results reported by Forster are particularly significant and have been widely quoted in connection with discussions embracing a consideration of the metabolic influence of water ingestion. Forster fasted a dog seven days and upon the eighth day increased the water ingestion of the animal 1828 cc. Under this augmented water intake the output of urea was increased from 12.14 grams for the seventh day to 22.91 grams for the eighth day. The total volume of water fed the dog upon the eighth day was 3000 cc, the entire volume of the fluid being fed within a half-hour interval. Rubner's experiments were also of interest in this connection. This investigator used rabbits as subjects, his experiments being so planned that the animals received no water for several days, and this total withdrawal of fluid was followed by a period when the animals were allowed water *ad libitum*. Under the influence

<sup>1</sup> Voit *Untersuchungen über den Einfluss des Kochsalzes, etc* München' 1860, p. 61

- Forster Quoted by Feder, *Zeitschr f Biol*, *xv*, p. 175, 1878

<sup>2</sup> Rubner *Zeitschr f Biol*, *xvii*, p. 221, 1881

<sup>4</sup> Fraenkel *Virchow's Archiv*, *lxxi*, p. 119, 1871



of the high water intake the output of urea was nearly double that excreted during the period when the animals were permitted no water. Voit also noted an increased urea output following a pronounced increase in the water ingestion of a fasting dog. In common with the other investigators mentioned Fraenkel observed that a high water intake was accompanied by an increased urea output. The increase in the case of Fraenkel's dog was very small, however, being but 0.79 gram of nitrogen for a twenty-four hour period.

#### DESCRIPTION

In the data reported by other investigators upon the influence of a copious water ingestion upon the fasting organism the increased volume of water has been introduced in every instance into the fasting subject in the *early days* of the fasting period. The experiments having been conducted many years ago the methods of analysis were of course rather inaccurate when judged according to modern standards. Urea was the only form of nitrogen which was determined with any degree of accuracy. Bearing the above facts in mind it seemed worth while to study the influence of water upon an animal which had reached the *advanced stages* of fasting and to follow the course of the excretion of the various nitrogenous urinary constituents.

The subject of the experiment was our fasting dog "Oscar" who ultimately succeeded in fasting 117 days as already reported.<sup>1</sup> The general plan of the experiment was the same as that employed in the other experiments already published from this laboratory.<sup>2</sup>

The weight of "Oscar" at the beginning of the fast was 26.33 Kg. He was brought into approximate nitrogen equilibrium on a diet containing 15.796 grams of nitrogen per day and made up of the following constituents: Meat, 400 grams, cracker dust, 100 grams, lard, 45 grams, bone ash, 12 grams, water, 700 cc. After reaching approximate nitrogen equilibrium the solid portion of the diet was withdrawn, the animal being fed the 700 cc of water by means of a tube, during each day of a 59-day fast. Dur-

<sup>1</sup> Howe, Mattill and Hawk. *Proc. Amer. Soc. Biol. Chem.*, 1, p. 260, 1910.

<sup>2</sup> Howe and Hawk. *Journ. Amer. Chem. Soc.*, 33, p. 215, 1911.

mg the sixtieth, sixty-first, sixty-second and sixty-third days the water ingestion was increased to 2100 cc this large volume being fed in four separate portions according to the following schedule 9 a m, 400 cc, 1 p m, 700 cc, 4 p m, 400 cc, 9 p m, 600 cc. The water ingestion was then reduced to the 700 cc level and continued at this point throughout the remainder of the experiment.

#### DISCUSSION OF RESULTS

The data of interest in connection with our report are given in Tables I to III, pp 422, 423. We have included in Table I the general data for a period of thirteen days embracing an interval of six days immediately preceding the time of increased water ingestion, a four-day period during which the water intake was increased 200 per cent above that customarily ingested, and an after period of three days.

*Total Nitrogen Excretion* For a period of six days previous to the interval during which the water ingestion was increased 200 per cent the average daily output of nitrogen had been 2 872 grams. The effect of the first day's intake of the 2100 cc of water was to raise this average to 5 097 an increase of 2 225 grams or 77.5 per cent. The total nitrogen values continued high for the second and third days, 3 690 and 3 096 grams, and upon the fourth day practically assumed the normal level with an excretion of 2 793 grams. If we take the four days of the water period into consideration we see that the excretion of nitrogen was increased by 3 188 grams during that time.

The course of the nitrogen excretion of this fasting dog under the influence of a copious water intake is seen to be similar to that observed in this laboratory<sup>1</sup> in experiments upon normally fed human subjects. The main points to which attention should be directed are the increased output of nitrogen upon the first day of high water intake followed by the diminution in the nitrogen and the subsequent assumption of a compensating subnormal level. If this experiment upon the dog be taken into consideration in connection with the other experiments above mentioned it will be observed, however, that the increase in the nitrogen output was much more

<sup>1</sup> Fowler and Hawk *Journ of Exp Med*, xii, p 388, 1910, Wills and Hawk. Unpublished.

pronounced in the fasting test. At first thought this fact might be construed as furnishing evidence in favor of the theory that the increased nitrogen following copious water ingestion is due to flushing out of the tissues rather than to a true stimulation of protein catabolism. However, we do not feel inclined to accept such an interpretation. Here we have an animal which has been receiving 700 cc of water each day for about two months. During this time the urine volumes have ranged from an average of 450 cc to 475 cc per day. Such being the case it is inconceivable to us that 2.225 grams of nitrogen or 77.5 per cent of that normally excreted could have been washed out from the tissues during a twenty-four hour period through the flushing properties of 1400 cc of water. We believe this increased nitrogen output to have arisen from the catabolism of protein material, the large volume of water being the active stimulatory factor which brought about this increased protein catabolism. Further arguments in substantiation of this belief will be given in a later paragraph.

*Urea Excretion* The output of urea-nitrogen in general ran parallel with that of total nitrogen. From a daily average value of 2.454 grams for the fore period it increased to 4.054 upon the first day of the water period and returned almost exactly (2.430 grams) to the pre-water level upon the third day. The urea nitrogen therefore assumed its normal course one day sooner than the total nitrogen. The percentage values are given in Table II, p 423.

*Ammonia Excretion* The average daily output of ammonia nitrogen for the six-day fore period was 0.261 gram. Upon the first day of the water period this value increased to 0.469 gram and remained high throughout the entire period, the values for the remaining days of the period being 0.362, 0.391 and 0.284 gram. This increase in the ammonia excretion under the influence of a high water ingestion is right in line with observations already reported from this laboratory on normally fed subjects<sup>1</sup>. In the work already published this augmentation of the ammonia excretion under the influence of copious water drinking was interpreted as indicating that the water had stimulated the flow of gastric juice and that the excess hydrochloric acid entering the intestine

<sup>1</sup> Fowler and Hawk. *Loc cit*, Wills and Hawk. *Proc Soc Biol Chem* June, 1911, Wilson and Hawk. Unpublished.

had subsequently united with ammonia formed in the process of protein deamidation and the ammonium chloride thus formed had at least in part been excreted as such instead of lending itself to the formation of urea in the liver. We therefore interpreted the increased ammonia output as indicative of a stimulated gastric function.

We see no reason for altering the above interpretation in order to explain the high ammonia nitrogen values observed in this fasting study. To be sure we have no protein ingested and we cannot therefore consider the ammonia to have arisen from the deamidation of *ingested* protein. However if we examine the figures for the excretion of total nitrogen during the water interval we will observe that these values are sufficiently far above the normal values to account for the catabolism of considerable *tissue* protein. We may therefore consider that the increased ammonia observed during the water period of the fasting experiment arose primarily through the deamidation of tissue protein in a manner analogous to the formation of the ammonia through the deamidation of food protein in the other experiments mentioned.

We are well aware of the "fasting acidosis" and have discussed it recently in connection with another article from this laboratory.<sup>1</sup> In the true fasting acidosis we have certain organic acids formed through the abnormal metabolic régime of fasting, whereas in the present instance we believe the acid concerned in bringing about the heightened ammonia output is the hydrochloric acid of the gastric juice. Then, too, fasting acidoses, particularly in experiments on men, generally increase progressively in severity and reach their maximum intensity in the closing stages of the fasting interval. If Table I, p 422, of the present paper be examined it will be noted that the high ammonia values for this fasting test can not be explained upon the basis of any such relationship. It will be observed that the normal ammonia value of 0.261 gram was nearly doubled upon the first water day (0.469) and that there followed a gradual *decrease* to the end of the period of high water ingestion. And furthermore as soon as the period of high water intake closed the decrease in the output of ammonia was very precipitate as is evidenced by the fact that the average daily output

<sup>1</sup> Howe, Mattill and Hawk *Journ Amer Chem Soc*, **xxiii**, p 568, 1911

TABLE I  
General Data

DAY OF FAST	BODY WEIGHT	VOLUME OF URINE	SPECIFIC GRAVITY	REACTION OF URINE	TOTAL N	UREA N	AMINO ACIDS N	CREATININE N	CREATININE N	PHOSPHORUS N	ALLANTOIN N	UNDETERMINED N
<i>Fasting—700 cc Water Per Day</i>												
	kg.	cc			grams	grams	grams	grams	grams	grams	grams	grams
54-57	16 34— 15 98	449	1006	acid	2 986	2 555	0 278	0 172		0 015	0 011	
58-59	15 90— 15 78	475	1005	acid	2 642	2 253	0 226	0 152		0 015	0 011	
<i>Fasting—2100 cc Water Per Day</i>												
60	16 09	1385	1002	acid	5 097	4 054	0 469	0 223	0 034	0 005	0 036	0 276
61	15 79	2390	1002	acid	3 690	2 980	0 362	0 174	0 071	0 004	0 024	0 075
62	15 89	1685	1001	acid	3 096	2 430	0 391	0 142	0 032	0 002	0 036	0 063
63	15 32	1840	1002	acid	2 793	2 203	0 284	0 138	0 045	0 002	0 023	0 093
<i>Fasting—700 cc Water Per Day</i>												
64	14 94	820	10035	acid	2 256	1 796	0 224	0 118	0 032	0 002	0 016	0 068
65	14 71	640	1004	acid	3 016	2 421	0 268	0 159	0 022	0 012	0 018	0 116
66	14 64	545	1005	acid	2 173	1 790	0 192	0 107	0 021	0 007	0 010	0 046

TABLE II  
Percentage Excretion in Terms of Total Nitrogen

DAY OF FAST	UREA	AMMONIA	CREATININE	CREATINE	PURINE	ALLANTOIN	UNDETERMINED
<i>Fasting—700 cc Water Per Day</i>							
54-57	85.57	9.31	5.76		0.50	0.37	
58-59	85.28	8.55	5.75		0.57	0.42	
<i>Fasting—2100 cc Water Per Day</i>							
60	79.54	9.20	4.38	0.67	0.10	0.71	5.41
61	80.76	9.81	4.71	1.92	0.11	0.65	2.03
62	78.49	12.63	4.59	1.03	0.06	1.16	2.04
63	78.88	10.17	4.94	1.61	0.07	1.00	3.33
<i>Fasting—700 cc Water per Day</i>							
64	79.61	9.93	5.23	1.42	0.09	0.71	3.01
65	80.27	8.89	5.27	0.73	0.40	0.60	3.85
66	82.37	8.84	4.92	0.97	0.32	0.46	2.11

TABLE III  
Body Weights, Creatinine Coefficient and Percentage Water Elimination

DAY OF FAST	BODY WEIGHT	GAIN OR LOSS IN WEIGHT PER DAY	PERCENTAGE GAIN OR LOSS IN WEIGHT PER DAY	CREATININE NITROGEN	CREATININE COEFFICIENT	URINE VOLUME	WATER INGESTION	PER CENT OF INGESTED WATER ELIMINATED THROUGH THE KIDNEYS
<i>Fasting—700 cc Water per Day</i>								
54-57	Lbs 15.98*	-0.12†	-0.75	mg% 172	10.8	cc 449	cc 700	64.12
58-59	15.78*	-0.10†	-0.63	152	9.6	475	700	67.83
<i>Fasting—2100 cc Water per Day</i>								
60	16.09	+0.31	+1.96	223	13.8	1385	2100	65.95
61	15.79	-0.30	-1.86	174	11.0	2390	2100	113.81
62	15.89	+0.10	+0.63	142	8.9	1685	2100	80.24
63	15.32	-0.57	-3.59	138	9.0	1840	2100	87.62
<i>Fasting—700 cc Water per Day</i>								
64	14.94	-0.38	-2.48	118	7.0	820	700	117.10
65	14.71	-0.23	-1.54	159	10.8	640	700	91.39
66	14.64	-0.07	-0.48	107	7.3	545	700	77.83

\* The body weight given is the weight at the end of the period

† Average loss per day

of ammonia-nitrogen for the three days following the period was but 0.228 gram, the final value being 0.192 gram. For the percentage values see Table II, p. 422.

*Creatinine Excretion* The average daily output of creatinine nitrogen for the six days preceding the period of high water intake was 0.165 gram. Upon the first day of the water period this value was increased to 0.223 gram and from this point to the end of the period the values decreased progressively, the final day showing an output of only 0.138 gram. As soon as the normal water level was again assumed the creatinine output sank still lower as is indicated by an average output of 0.128 gram for three days, the output for the third day being but 0.107 gram.

If the data for the excretion of creatinine-nitrogen by normally nourished individuals under the influence of high water intake<sup>1</sup> be examined it will be noted that there is in every instance a *decreased* output. Likewise if the data from certain fasting tests<sup>2</sup> be consulted the trend will also be noted to be *downward* in those experiments. In the present paper, however, we have submitted data which indicate a 30 per cent *increase* upon the day in which the fasting organism was first subjected to the influence of the high water ingestion. In other words we have data indicating that when an animal organism is subjected to the influence of either *water drinking* or *fasting* that a *decreased* output of creatinine results whereas when the *two* agencies (water drinking and fasting) are brought to bear simultaneously upon an organism a pronounced *increase* in the creatinine output occurs, particularly upon the first day of the test. It must be remembered in this connection that so far as the effect of the water is concerned, in one instance we came up to the period of high water ingestion from a fasting level whereas in the other instance we approached it from the level of normal nutrition. This observation of an initial increase in the creatinine output followed by a subsequent decrease may be interpreted according to Lefmann<sup>3</sup> as a definite indication of a stimulated catabolism of protein material.

In a simple fasting test where the fast follows directly after a period of normal feeding and no extraneous agencies such as water

<sup>1</sup> Fowler and Hawk *Loc. cit.*, Howe and Hawk Unpublished

<sup>2</sup> Howe and Hawk *Loc. cit.*, Howe, Mattill and Hawk *Loc. cit.*

<sup>3</sup> Lefmann *Zeitschr. für physiol. Chemie*, lvii, 476, 1908

drinking are included we would expect a decreased output of creatinine if we accept the current theory<sup>1</sup> that the creatinine excretion is a function of the amount of active muscular tissue in the body. In the same way we may logically expect a lowered output of creatinine to result from the increased protein catabolism<sup>2</sup> accompanying the ingestion of a large volume of water by a normally nourished individual<sup>3</sup>. The data in the present paper indicate clearly that there was probably a very pronounced stimulation of protein catabolism under the influence of the high water intake. Therefore, in line with the suggestion of Lefmann we would naturally look for a *decreased* output of creatinine upon the days in which copious quantities of water were ingested. Instead of a decreased creatinine excretion, however, we observe that the output of this urinary constituent was increased. It seems evident, therefore, that the factors making for a decreased output of creatinine under the influence of water drinking are more than counterbalanced by other factors which are brought into play when to the influence of the water is added the abnormal metabolic regime of fasting. For percentage values see Table II, p 423.

*Creatine Excretion* The data given in Table I, p 422 indicate that there was no creatine in the urine for the six-day period preceding the time of high water ingestion. Other data on file in this laboratory<sup>4</sup> indicate that for a period of twelve days before the water interval only 0.010 gram of creatine nitrogen was excreted and that during an interval of eight days before the water period the urine of the dog was practically creatine-free. At the opening of the water period when the daily ingestion of water was increased from 700 to 2100 cc creatine appeared and continued to be excreted upon every day of the water period. The daily output of creatine-nitrogen during the water period ranged from 0.032 to 0.071 gram, the total weight of this form of nitrogen excreted under the influence of the water being 0.182 gram.

The failure to find any creatine in the urine of this fasting dog from the fifty-second to the fifty-ninth days of fasting inclusive

<sup>1</sup> Schaffer *Amer Journ of Physiol*, **xvi**, p 252, 1906, *Folin Ibid*, **xiii**, p 66, 1905

<sup>2</sup> Lefmann *Loc cit*

<sup>3</sup> Fowler and Hawk *Loc cit*

<sup>4</sup> Howe, Mattill and Hawk Unpublished



was a rather surprising observation. This condition of affairs afforded us, however, an excellent opportunity for studying the stimulatory powers of water as regarded the creatine output. It is a very significant finding that the urinary creatine-nitrogen values should increase from zero to 0.182 gram in the course of four days solely through the influence of an increase of 1400 cc in the daily water ingestion.

Our creatine nitrogen and total nitrogen data furnish additional evidence in favor of the hypothesis that creatine is held in such a combination within the muscle that its release through the influence of water "is not necessarily accompanied by the disintegration of the other nitrogenous muscle constituents which go to form the total nitrogen quota of muscular tissue."<sup>1</sup> As before mentioned, the total nitrogen output during the water period was increased 3.188 grams above the normal level. If we calculate on the basis of a nitrogen value of 3.25 per cent for flesh we find that the 3.188 grams of nitrogen is equivalent to 98 grams of flesh. In like manner taking 0.123 per cent for the creatine-nitrogen value of flesh we learn that the creatine output for the water period represents 148 grams of flesh. However if this 148 grams of flesh had been completely disintegrated our total nitrogen figures should be higher, actual calculation showing a discrepancy of about 34 per cent. This fact furnishes an apparently logical basis for the claim that a portion of the creatine excreted under the influence of water was removed from muscular tissue whose total nitrogen quota was not excreted inasmuch as the muscle continued to function within the organism. See p. 428 for further discussion of this form of calculation.

As already discussed in a previous paper from this laboratory the data for the creatine output following water drinking furnish an important substantiation for the hypothesis that the increase in the total nitrogen excretion which follows water drinking is due to a true stimulation of protein catabolism rather than to a mere flushing of the tissues. See Table II, p. 423 for percentage values.

After the foregoing data had been sent to the printer our attention was called to an article by Mendel and Rose in the October number of this

<sup>1</sup> Fowler and Hawk *Loc. cit.*, Howe and Hawk *Loc. cit.*

Journal<sup>1</sup> which criticised some points in regard to the creatine-creatinine interpretation of a former paper from this laboratory<sup>2</sup>

It had been found by us that the muscular tissue of a dog subjected to repeated fasting contained much less creatine than did the muscular tissue of normal dogs. It was concluded from this that a portion of the creatine content of such muscle had been withdrawn and excreted leaving the partially decreatinized muscle "still functioning as living tissue within the body of the animal"<sup>3</sup> To this interpretation Mendel and Rose reply "It is difficult to understand how any such withdrawal of creatine from functioning muscle could occur. Urano<sup>4</sup> has shown that muscle creatine is held in a non-diffusible form and is probably loosely combined with the muscle protoplasm. Its liberation would only be possible after complete disintegration of the muscle bundles." To our mind this finding of Urano, which so far as we are aware awaits verification, cannot justly be considered as opposed to our finding of a lowered creatine value for the muscular tissue of a dog subjected to repeated fasting. Urano was working with so-called surviving muscle which was, of course, severed from all its circulatory and nervous relations and completely removed from the influence of the many and varied metabolic processes which are so intimately associated with living tissues and which unfortunately are probably as yet but imperfectly understood. On the other hand in the case of our dog we were dealing with muscle which was functioning normally within the organism. As Mendel has very aptly said in a recent article,<sup>5</sup> in connection with a discussion of the experiments of Urano and others, "*We must bear in mind that all experiments such as those just reported are conducted under artificial conditions different from what pertains in normal muscular activity*"

The finding by Dorner<sup>6</sup> of a lowered creatine value for the muscular tissue of fasting rabbits, as well as the observation by Mellanby<sup>7</sup> to the effect that human carcinomatous tissue possesses a sub-normal creatine value furnish important confirmatory evidence in favor of the reasonableness of the hypothesis as to decreatinized muscle.

Mendel and Rose next proceed to take exception to our mathematical demonstration that the total nitrogen excretion is far more than sufficient to account for the creatine output if we consider that urinary creatine represents completely and permanently disintegrated muscular tissue.<sup>8</sup> The idea of making a calculation of this sort suggested itself after we had noted a similar calculation in the article by Mellanby to which reference has already been made. It may be well to mention the fact that the workers in

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<sup>1</sup> Page 262

<sup>2</sup> Howe and Hawk. *Loc cit*

<sup>3</sup> Howe and Hawk. *Loc cit*, p 253

<sup>4</sup> Urano. *Hofmeister's Beiträge*, ix, p 104, 1907

<sup>5</sup> Mendel. *Science*, N. S., xlix, p 584, 1909

<sup>6</sup> Dorner. *Zeitschr f physiol Chem*, lvi, p 225, 1907

<sup>7</sup> Mellanby. *Journ of Physiol*, xxxvi, p 447, 1908

<sup>8</sup> A similar calculation is given on p 426 of the present paper

this laboratory have never considered that they were concerned with *exact* methods when they attempted to show relationship or lack of relationship by means of a mathematical procedure such as that mentioned above. Calculations of this nature however no doubt possess value in that they may possibly bring out relationships which would not be apparent unless a calculation of this sort were made.

The scientific limitations of this method of procedure have been duly appreciated. The great abundance of uncontrolled factors would, of course, render definite deductions out of the question. As indicating our ideas as to the limitations of this form of calculation permit us to make citations from a recent paper from this laboratory.<sup>1</sup> "In any attempt to show a mathematical relationship between the urinary total purine nitrogen and the total nitrogen output as given in an earlier paragraph, it must be borne in mind that the flesh of different portions of the same animal will vary in their purine values." And further along in the same paragraph we read "There is obviously no possible way of estimating what percentage of this increased output is due to the disintegration of the one type of tissue as distinct from the others." Fortunately the calculations of this character made in connection with our experimental work on creatine excretion do not form any material part of the basis for our interpretations.

In connection with the discussion of our calculation procedure Mendel and Rose call attention to the fact that Paton<sup>2</sup> has suggested a possible resynthesis of nitrogen under such conditions. We were aware of Paton's contention and discussed it as follows on p. 239 of the article criticised by Mendel and Rose. "At the same time we must not lose sight of the fact that the muscular tissue whose nitrogen store is seemingly intact at the end of a fast may assume this condition not because the catabolic processes have not been sufficiently profound to alter its inner structure but rather because of certain synthetic reactions which have followed or accompanied the primary disintegration of the tissues." They speak further of the "enormous store of reserve nitrogen" possessed by dogs and of its influence upon the calculation mentioned. This "reserve nitrogen" if amenable to estimation should, of course, be subtracted from the total nitrogen value obtained by us. This procedure would naturally tend to augment the discrepancy which we have already emphasized (p. 426) as existing between the total nitrogen and creatine nitrogen data of the present paper. This question of "reserve nitrogen" certainly does not introduce any appreciable inaccuracy into the calculation given on p. 426 of the present paper inasmuch as the nitrogen reserve of this dog must have been a negligible quantity after a fast about *two months* in length.

It is an interesting and significant fact that notwithstanding the many possible sources of error surrounding the attempt to show a mathematical relationship between the total nitrogen and creatine-nitrogen data in the repeated fast referred to by Mendel and Rose, that there was "a direct

<sup>1</sup> Wreath and Hawk *Journ Amer Chem Soc*, xxxiii, p. 1620, 1911

<sup>2</sup> Paton *Journ of Physiol*, xxxix, p. 485, 1910

relationship between the amount of nitrogenous material catabolized and the creatine excreted For example with practically the same amount of nitrogen excreted under the same conditions, i e, two fasts continuing to the premortal rise, and with two equal but decidedly different rates of nitrogen excretion, practically the same amount of creatine was excreted"<sup>1</sup> It is also worthy of note that the creatinine output of the second fast was about 64 per cent greater than that of the first fast although the creatine output was wonderfully uniform as before mentioned This increase in creatinine was due simply to the fact that the second fast was twice as long as the first fast

But according to Mendel and Rose the "greatest fallacy" in our reasoning is that we "entirely neglect to consider the output of preformed creatinine in their (our) calculations" They then state that on the basis of the *total creatinine* data (creatinine + creatine) "the muscle waste" in the *second* fast of the dog used in our experiment "is more than sufficient to account for all urinary nitrogen" That is true However, if we accept this suggestion offered by Mendel and Rose and make our calculation on the *total creatinine* basis we secure additional evidence in favor of the hypothesis of decreatinized muscle, already mentioned

In connection with the finding by Mendel and Rose of an *increased* creatine content in the muscles of fasting hens and rabbits it should be borne in mind that in our experiments the tissue which showed the *low creatine* value was the tissue of a dog whose general metabolic processes had been strikingly altered through the medium of a "repeated" fast It may very well be therefore that the muscles of a dog subjected to "repeated" fasting may exhibit characteristics strikingly different from the muscles of hens and rabbits which have been subjected to but a *single* fast

*Purine and Allantoin Excretion* The data regarding these forms of nitrogen were obtained by Mr S R Wreath and have already been published in another connection<sup>2</sup> Because of their interesting relationships to the other data given in the present paper we will give a brief summary of the findings

It will be noted that during the period of copious water ingestion there was a marked increase in the output of allantoin-nitrogen and a much more pronounced decrease in the purine-nitrogen output These findings were interpreted as indicating that "the high water ingestion had caused sufficient stimulation of the oxidative processes of the body to bring about the oxidation into allantoin of such substances as would ordinarily go to make up the purine nitrogen quota This oxidation was almost quantitative during

<sup>1</sup> Howe and Hawk p 240

<sup>2</sup> Wreath and Hawk *Journ Amer Chem Soc*, **xxiii**, p 1601, 1911

the latter part of the water period. During this interval only 0.07 per cent of the nitrogen of purine origin was excreted in the form of purine-nitrogen. The finding of a lowered output of purine nitrogen was in full agreement with the finding already reported from this laboratory<sup>1</sup> of a lowered output of uric acid under the influence of copious water drinking."

"It was further found that the *total purine* values (sum of the allantoin-nitrogen and purine-nitrogen) for the four days of increased water ingestion were higher than the total purine values for any other four-day period during the fast of 117 days. This finding was interpreted as indicating that the high water intake had caused a stimulation of protein catabolism." By proper calculation it was then determined that "provided sufficient flesh was catabolized to yield the increased output of total nitrogen eliminated during the water period, 46 per cent of the purine nitrogen of this quantity of flesh was unaccounted for. This quota of purine-nitrogen might have been oxidized to stages below allantoin and thus escaped determination."

In common with the creatine data we submit the total purine data as further evidence in favor of the hypothesis that the increased output of total nitrogen following a high water intake is due to the stimulation of protein catabolism rather than to a flushing out of the tissues.

*Miscellaneous Considerations* In table III, p. 423, are given the data on body weights, creatinine coefficient and the percentage elimination of water. The body weights can have but slight significance inasmuch as during the water period the retention of water would cause fluctuating variations.

The creatinine coefficient was considerably higher during the first two days of the water period than during the fore period. The values were in general lower during the after period. The high values in the first part of the water period were due directly to the influence of the water in augmenting the creatinine output whereas the low values of the after period may be ascribed to the fasting régime.

The percentage of the ingested water which was eliminated through the kidneys was higher during the water and after periods than during the fore period. In reports formerly made by one of

<sup>1</sup> Rulon and Hawk. *Journ Amer Chem Soc*, 58, 1686, 1910

us,<sup>1</sup> it has been shown that when large volumes of water are introduced into a normally fed organism a higher percentage of the ingested amount is excreted through the kidneys than when small volumes of water are fed. A similar finding has been reported by us<sup>2</sup> in connection with fasting men. This relation does not hold true during the post-fasting period of regeneration inasmuch as the retention of water by the tissues during this period tends to make comparative data unreliable. For example in the experiment just mentioned, only 15.4 per cent of a 3566 cc water ingestion was eliminated upon the second day of the regeneration period whereas 35.9 per cent of a 2424 cc water ingestion was eliminated upon the following day.

In general the data for the water elimination of the present investigation coincide with those previously presented. There was for example a higher percentage of the ingested water excreted during the period of high water intake than during the fore period of low water level. The high percentage values of the final period are of course due to a "water lag" or retention of water by the tissues.

#### SUMMARY

A fasting dog ("Oscar") weighing 26.33 kg was subjected to a fifty-nine-day fast receiving each day 700 cc of water by means of a tube. On the sixtieth day and for the three following days the water ration was increased 200 per cent and at the end of this water period the former water level (700 cc) was again assumed.

Determinations of total nitrogen and of nitrogen in the form of urea, ammonia, creatinine, creatine, purine and allantoin were made. The analytical data indicated that the water had brought about an increase of 77.5 per cent in the total nitrogen output for the first day of the period, the course of the excretion decreasing from this point to the end of the period, the value for the final day being about normal.

The excretion of nitrogen in the form of ammonia was markedly increased during the period of high water intake, a feature which

<sup>1</sup> Hawk, *Univ of Penn Med Bull*, VIII, p 7, 1905, Rulon and Hawk, *Arch of Int Med*, VII, p 536, 1911.

<sup>2</sup> Howe, Mattill and Hawk, *Loc cit*

was interpreted as indicating that the large volume of water had caused a pronounced stimulation of the gastric function

The output of nitrogen in the form of creatinine was found to undergo a sharp *increase* upon the initial day of the water period. This point is significant in view of the former finding of a *decreased* output of creatinine when a normally fed organism is subjected to the influence of water drinking or of fasting. In the present instance the two agencies were working *simultaneously*.

For a period of eight days preceding the water period the urine of the dog had been practically *creatine-free*. With the inception of the interval of high water intake creatine in considerable quantity appeared in the urine and continued to appear throughout the entire period.

The copious water ingestion was found to produce a pronounced *increase* in the allantoin-nitrogen elimination and a more than compensating *decrease* in the excretion of purine-nitrogen. The low purine values are believed to be due to the fact that certain oxidative processes have been so stimulated as to oxidize a certain quota of the purine-nitrogen precursors into allantoin. The total purine values (allantoin + purine) were *increased* during the water period.

The total nitrogen values when taken into consideration in connection with the values for creatine-nitrogen and total purine nitrogen are believed to furnish strong substantiation for the hypothesis that the increased nitrogen output associated with increased water ingestion is due to a true stimulation of protein catabolism rather than to a flushing of the tissues.

# STUDIES IN NUTRITION

## IV THE UTILIZATION OF THE PROTEINS OF THE LEGUMES

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### EARLIER STUDIES

The literature on this subject has been so adequately reviewed by Wait, that only the most cursory consideration of the earlier work need find place here. In experiments on a man, Hoffmann found the nitrogen of a diet of lentils, bread and potatoes to be 53 per cent available, against a utilization of 82 per cent for the nitrogen of meat. Woroschiloff compared the utilization of the protein of peas with that of meat protein. In three cases the meat protein was 90, 92, and 96 per cent utilized against 83, 88, and 90 per cent for the digestibility of the protein of the peas. Strümpell found the nitrogenous constituents of "leguminose"—a finely ground commercial preparation, consisting of a mixture of lentils, peas and rye—to be 90 per cent available, against a utilization of



but 60 per cent in an experiment with unground lentils. Rubner has pointed out that in the experiments of both Woroschiloff and Strumpell, materials other than legumes were eaten, and these accessories may have exerted a favorable influence. Accordingly, Rubner conducted two experiments with thoroughly cooked hulled peas which were the only food consumed. The utilization was 72 to 83 per cent. In Malfatti's experiments, peas were 86 per cent utilized and Potthast found lentils to be 74 per cent digested. In an experiment by Prausnitz, white beans, soaked for several hours and then cooked till soft, yielded 70 per cent available nitrogen. Erismann found the nitrogen of peas to be 80 per cent digested. Richter obtained a utilization of 90 per cent for the nitrogen of peas cooked in distilled water, against 83 per cent when hard water was employed in the process of cooking. Under the latter condition particles of apparently unchanged peas were observed in the feces. The poor digestibility of the peas cooked in hard water is attributed in part to the formation of difficultly digestible alkali earth albuminates, and in part to the digestive disturbance due to the magnesium salts in the water. Snyder reported a utilization of 80 per cent for the protein of peas, and obtained a similar result with beans. In their experiments with the Maine lumbermen, Woods and Mansfield estimated the protein of beans to be at least 78 per cent utilizable, and an average digestibility of 65 per cent is reported in Oshima's compilation of Japanese investigations. In a very thorough study, Wintgen found the average coefficients of digestibility of lentils, beans, and peas to be 78, 80 and 86 per cent respectively. Wintgen's results are in accord with those obtained in an extensive investigation by Wait, in which a utilization of 77 to 78 per cent was obtained for bean protein, and 70 to 83 per cent for cow pea protein.

In commenting upon this literature one can but reiterate the statements made in a previous paper<sup>1</sup> of this series, and point out the necessity for studying the utilization of the isolated protein, or material in which the protein is more readily accessible to the digestive juices.

<sup>1</sup> Mendel and Fine. *This Journal*, x, p 303, 1911

## EXPERIMENTAL PART

*Products Employed*

1 **SOY BEAN**<sup>1</sup> This material was an impalpable yellow powder, which betrayed no cellular structure under the microscope. In respect to consistency, it would thus appear to be ideal for digestion experiments. As may be observed from the accompanying analysis,<sup>2</sup> the soy bean offers several points of interest

	<i>per cent</i>
Protein	44.6
Fat	19.4
Cane sugar	9.3
Mineral matter	4.2
Crude fiber	2.3
Moisture	5.3
Non-nitrogenous extract	14.8

Its content of protein and fat far exceeds that of any other legume, which condition seems to have been appreciated in Japan, for, according to Oshima, it is next to rice in importance in the Japanese dietary.<sup>3</sup> In addition to cane sugar, the presence of galactans and of pentosans has been detected by Schulze and his collaborators.<sup>4</sup> The soy bean does not give the ordinary iodine test for starch.<sup>5</sup>

2 **WHITE BEAN** This was the ordinary white bean of commerce.

3 **CRUDE BEAN PROTEIN** Experiments with the ordinary white bean are subject to the same criticism as has been offered in connection with the work of previous investigators. The attempt was here made to thoroughly rupture the cells and dissolve and wash away the starch. The method in brief was as follows: about

<sup>1</sup> Mr. M. F. Deming of the Cereo Company, Tappan, N. Y., very kindly contributed this material.

<sup>2</sup> Reported by Ruhrah. *Journal of the American Medical Association*, LV, p. 1664, 1910.

<sup>3</sup> Oshima, (see bibliography) gives an interesting account of the various soy bean preparations, which are common articles of diet in Japan.

<sup>4</sup> For the literature see Schulze and Godet. *Zeitschrift für physiologische Chemie*, LV, p. 279, 1909.

<sup>5</sup> Cf. Oshima *loc cit* p. 26.

5 pounds of finely ground hulled beans<sup>1</sup> were mixed with water and heated in a glycerol bath. After the mixture had been held near 100° C for about an hour, the thin mush which had formed was cooled below 75° C and a glycerol extract of malt diastase added as a result of which, after a few minutes, starch could no longer be detected with iodine in a test-tube trial. The material thus obtained was washed by decantation and the water driven off by heat until about 20 per cent was made up of solid matter. The resulting preparation was a thick mush, which could be conveniently pressed into cakes and preserved frozen. Although no iodine test for starch was obtained in a test-tube trial, nevertheless, when a sample treated with this reagent was examined under the microscope, not infrequently starch grains were observed within cells, which had apparently not in any way been affected by the treatment to which they had been subjected. This insufficient rupture undoubtedly accounts for the incomplete conversion of the starch.

*Analysis of Crude Bean Protein (calculated for anhydrous material)*

	per cent
Protein ( $N \times 6.25$ )	51.1
Sugar from insoluble carbohydrate (by hydrolysis)	28.9
Sugar from soluble carbohydrate (by hydrolysis)	2.4
Ash	2.6
Ether extract*	4.0
Crude fiber (by difference)	11.0

\*Estimated from Atwater and Bryant. U. S. Department of Agriculture. Bull. 28 (Revised), p. 65, 1906. Material was not available for analysis.

Attention is called to the fact that a barley protein preparation<sup>2</sup> with approximately the same concentration of protein contained practically no cellular structure or starch, yet yielded 20 per cent of carbohydrate by hydrolysis. The latter was believed to be hemicelluloses. It is thus probable that a not inconsiderable portion of the "carbohydrate by hydrolysis" of the above analysis was in reality also made up of hemicelluloses.<sup>3</sup>

<sup>1</sup> Furnished by Mr. Deming who also prepared a considerable portion of the crude bean protein for us according to the method outlined.

<sup>2</sup> Mendel and Fine. *This Journal*, v, p. 340, 1911.

<sup>3</sup> At the time of proof reading we learn through a private communication from Prof. E. Schulze that hulled beans, *phaseolus vulgaris*, contain 12.9 per cent hemicellulose. We estimate the hemicellulose concentration of our preparation at approximately 25 per cent.

4 PHASEOLIN This material was very kindly furnished by Dr T B Osborne It was dried, ground to an impalpable powder, and found by analysis to contain 13 per cent of nitrogen

5 PEA GLOBULIN This material was prepared as follows dried peas were finely ground and repeatedly extracted with 10 per cent NaCl solution The perfectly clear extract thus obtained was saturated with ammonium sulphate, the resulting precipitate being collected on a filter paper, suspended in a small amount of water to which toluene had been added and dialyzed for about two weeks, that is, until free from sulphates Part of the preparation was obtained by dialyzing the saline extract, thus avoiding the necessity of the precipitation with ammonium sulphate The resulting precipitate was dried at 40° to 50° C, ground to an impalpable powder and found by analysis to contain 16 per cent of nitrogen

### *Metabolism Experiments*

SOY BEAN Man,<sup>1</sup> Table 1 The ordinary routine was followed a fore period (preceded by a three day adjustment period), during which a mixed diet was consumed, experimental period, in which over 90 per cent of the nitrogen ingested was furnished by soy bean, and an after period essentially like the fore period The character of the diet is outlined below

*Character of Diet*

	PRELIMINARY AND FORE PERIODS	EXPERIMENTAL PERIOD	AFTER PERIOD
	Daily Averages	Daily Averages	Daily Averages
	<i>grams</i>	<i>grams</i>	<i>grams</i>
Cracker	70		70
Egg	100		200
Peanut butter	75		
Meat	140		190
Soy bean		165	
Potato	100		120
Tomato	250	375	200
Apple	200	200	200
Orange	180	180	180
Milk	60	60	60
Sugar	130	140	130
Butter	50	100	90
Cereal coffee, tea	600		600

<sup>1</sup> The subject was one of us (M S F) twenty-four years of age, leading the usual active life of the laboratory

As will be observed, during the experimental period the cracker, egg, meat, and nut butter were completely replaced by soy bean, which furnished 91 per cent of the total nitrogen intake of this period. The daily nitrogen and calorific intakes in these periods were fairly constant, averaging about 12.6 grams and 2500 calories, respectively. The soy bean was boiled in water for one-half hour, salted to taste, and the tomatoes thoroughly incorporated into the resulting mush. The palatability of the mixture was still further increased by the addition of a very small amount of paprika. On the whole it may be said that this fare proved quite agreeable, no unpleasant symptoms appearing throughout the period of six days.

TABLE 1

*Soy Bean*

SUBJECT MAN Weight at beginning 56.8 kg Weight at end 56.6 kg	PERIOD I (5 days) Mixed Diet	PERIOD II (6 days) Soy Bean	PERIOD III (5 days) Mixed Diet
Composition of daily diet	Meat, egg, nut butter, potato, fruit, etc	Soy bean, fruit, etc, 90.5 per cent total nitrogen supplied by soy bean	Meat, egg, potato, fruit, etc (No nut butter)
	Estimated calories 2400	Estimated calories 2400	Estimated calories 2600
	Daily Averages	Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm	9.56	10.81	9.69
Total nitrogen, gm	11.11	12.72	11.16
Nitrogen in food, gm	12.78	12.93	12.22
Nitrogen balance, gm	+1.67	+0.21	+1.06
<i>Feces</i>			
Weight air dry, gm	25.0	26.6	25.2
Nitrogen, gm	1.55	1.91	1.47
Nitrogen, per cent	6.18	7.15	5.88
Nitrogen utilization, per cent	87.9	85.3	88.0

The subject felt in excellent condition throughout the entire experiment. Defecation took place regularly every morning and no diarrhoea occurred.

It will be observed from Table 1, that the *soy bean nitrogen is distinctly (if only slightly) less well utilized than that of the preceding and succeeding mixed diets*. The nitrogen concentration of the feces of the soy bean period is higher than in any other experiment on this subject which indicates that some soy bean protein escaped absorption.

**SOY BEAN Dogs (with agar and bone ash)**—Tables 2 to 4  
Dog 1, Table 2, was fed with a mixture of soy bean, lard, agar, bone ash and water. It was heated on the water bath for four to six hours, the purpose being to thoroughly "hydrate" the material, which, as fed to the animal, was a thick mush.

Dogs 5 and 7, Tables 3 and 4, were fed with similar ingredients and sugar in addition. The mixture, including the water, was not heated, but allowed to stand over night, after which the material appeared to be thoroughly "hydrated."

The plan of experimentation differed in no particular from those previously followed, and we may therefore proceed directly to an examination of the tables, which contain all the essential details.

*In the dog, the soy bean was in every case strikingly less well utilized than the meat fed under similar experimental conditions, and one also notes the persistently higher nitrogen concentration of the feces of the soy bean periods as compared with that of the meat-feces.* This is an indication, as noted above, that some soy bean protein has probably escaped digestion.

## Utilization of Legume Proteins

TABLE 2

*Soy Bean with Agar and Bone Ash*

SUBJECT DOG 1 Weight at beginning 15.0 Kg Weight at end 14.6 Kg		PERIOD III (5 days) Meat Feeding	PERIOD IV† (5 days) Soy Bean Feeding	PERIOD V (4 days) Meat Feeding
Composition of daily diet		grams	grams	grams
Composition of daily diet	Meat	300	Soy bean	147
	Lard	60	Lard	60
	Agar*	5	Agar	5
	Bone ash	15	Bone ash	15
	Water	300	Water	500
		Estimated calories 1070	Estimated calories 1110	Estimated calories 1070
Nitrogen output		Daily Averages	Daily Averages	Daily Averages
Urine nitrogen, gm		10 07	9 25	8 81
Total nitrogen, gm		10 60	10 94	9 38
Nitrogen in food, gm		10 38	10 44	10 44
Nitrogen balance, gm		-0 22	-0 50	+1 06
<i>Feces</i>				
Weight air dry, gm		29 4	55 6	29 5
Nitrogen, gm		0 53	1 69	0 57
Nitrogen, per cent		1 79	3 05	1 95
Nitrogen utilization, per cent		95 0	83 8	94 5

\* On the first two days of the period the indigestible was represented by 20 grams bone ash. This produced brittle feces hence in the remaining three days agar and bone ash were employed as noted in the table.

† Forced feeding necessary throughout the period—no vomiting.

TABLE 3

*Soy Bean with Agar and Bone Ash*

SUBJECT DOG 5 Weight at beginning 5.2 Kg Weight at end 5.2 Kg		PERIOD VI (4 days) Meat Feeding	PERIOD VII* (4 days) Soy Bean Feeding	PERIOD VIII (5 days) Meat Feeding
Composition of daily diet		grams	grams	grams
Composition of daily diet	Meat	150	Soy bean	69
	Sugar	20	Sugar	20
	Lard	20	Lard	25
	Agar	3	Agar	3
	Bone ash	7	Bone ash	7
		Water 100	Water 200	Water 100
		Estimated calories 510	Estimated calories 570	Estimated calories 510
Nitrogen output		Daily Averages	Daily Averages	Daily Averages
Urine nitrogen, gm		3 71	3 01	4 09
Total nitrogen, gm		4 11	4 26	4 44
Nitrogen in food, gm		4 93	4 90	4 80
Nitrogen balance, gm		+0 82	+0 64	+0 36
<i>Feces</i>				
Weight air dry, gm		15 5	34 0	15 0
Nitrogen, gm		0 40	1 25	0 35
Nitrogen, per cent		2 60	3 67	2 32
Nitrogen utilization, per cent		91 8	74 5	92 7

\* Cystitis developed but was cured in the course of two days by means of  $\text{AgNO}_3$  solution.

TABLE 4  
*Soy Bean with Agar and Bone Ash*

SUBJECT DOG 7 Weight at beginning 4.9 Kg Weight at end 4.6 Kg	PERIOD V (3 days) Meat Feeding	PERIOD VI (6 days) Soy Bean Feeding	PERIOD VII (5 days) Meat Feeding
Composition of daily diet	Meat 100	Soy bean 47	Meat 100
	Sugar 20	Sugar 20	Sugar 20
	Lard 20	Lard 25	Lard 20
	Agar 3	Agar 3	Agar 3
	Bone ash 7	Bone ash 7	Bone ash 7
	Water 100	Water 175	Water 100
	Estimated calories 430	Estimated calories 490	Estimated calories 430
	Daily Averages	Daily Averages	Daily Averages
Nitrogen output			
Urine nitrogen, gm	2.63	2.38	2.55
Total nitrogen, gm	2.89	3.04	2.79
Nitrogen in food, gm	3.29	3.34	3.20
Nitrogen balance, gm	+0.40	+0.30	+0.41
Feces			
Weight air dry, gm	12.7	20.5	12.8
Nitrogen, gm	0.26	0.66	0.24
Nitrogen, per cent	2.04	3.22	1.86
Nitrogen utilization, per cent	92.1	80.2	92.6

**SOY BEAN** *Dogs (without agar and bone ash)—Tables 5 to 13*  
 These experiments were conducted in essentially the same manner as those just reported, except that the indigestible adjuvants—agar and bone ash—were omitted. Tables 8 to 10 contain the results of trials instituted after the intestinal tract had been subjected to a thorough treatment with indigestible non-nitrogenous materials, the purpose being to remove as far as possible the accumulated intestinal debris.

Proceeding directly to a study of the tables, we again note the poor utilization of the soy bean nitrogen. A fuller discussion of these data with a consideration of the attending conditions will be offered below<sup>1</sup> in connection with the discussion of the results obtained with the crude bean protein.

In Oshumra's compilation one notes that certain soy bean products (e.g. tofu) are as much as 96 per cent utilizable. Tofu, however, is probably of an albumose nature and such favorable results should be correspondingly interpreted.



## Utilization of Legume Proteins

TABLE 5

*Soy Bean without Agar or Bone Ash*

SUBJECT DOG 5 Weight at beginning 5.9 kg Weight at end 6.0 kg		PERIOD XX (4 days) Meat Feeding	PERIOD XXI* (5 days) Soy Bean Feeding
Composition of daily diet		grams	grams
	Meat	150	Soy bean 64
	Sugar	25	Sugar 20
	Lard	20	Lard 20
	Water	100	Water 220
	Estimated calories 530	Estimated calories 530	
		Daily Averages	Daily Averages
Nitrogen output			
Urine nitrogen, gm	4 08	3 96	
Total nitrogen, gm	4 30	4 66	
Nitrogen in food, gm	4 59	4 61	
Nitrogen balance, gm	+0 29	-0 05	
Feces			
Weight air dry, gm	4 5	17 8	
Nitrogen, gm	0 22	0 70	
Nitrogen, per cent	4 95	3 90	
Nitrogen utilization, per cent	95 2	85 0	

\* One-quarter to one-half of the food forced each day

TABLE 6

*Soy Bean without Agar or Bone Ash*

SUBJECT DOG 6 Weight at beginning 6.1 kg Weight at end 6.3 kg		PERIOD XVI (4 days) Meat Feeding	PERIOD XVII (5 days) Soy Bean Feeding
Composition of daily diet		<i>grams</i> Meat 150 Sugar 25 Lard 20 Water 100 Estimated calories 530	<i>grams</i> Soy bean 64 Sugar 20 Lard 20 Water 220 Estimated calories 530
		Daily Averages	Daily Averages
	<i>Nitrogen output</i>		
	Urine nitrogen, gm	3 55	3 42
	Total nitrogen, gm	3 77	4 16
Nitrogen in food, gm	4 59	4 61	
Nitrogen balance, gm	+0 82	+0 45	
<i>Feces</i>			
Weight air dry, gm	3 5	18 2	
Nitrogen, gm	0 22	0 74	
Nitrogen, per cent	6 34	4 04	
Nitrogen utilization, per cent	95 2	84 0	

TABLE 7

*Soy Bean without Agar or Bone Ash*

SUBJECT DOG 7 Weight at beginning 5.9 Kg Weight at end 6.3 Kg		PERIOD XX (4 days) Meat Feeding	PERIOD XXI (5 days) Soy Bean Feeding
Composition of daily diet	Meat	grams 150	Soy bean 64
	Sugar	25	Sugar 25
	Lard	20	Lard 20
	Water	100	Water 225
	Estimated calories	530	Estimated calories 530
		Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm		3 55	3 41
Total nitrogen, gm		3 74	4 21
Nitrogen, in food, gm		4 59	4 61
Nitrogen balance, gm		+0 85	+0 40
<i>Feces</i>			
Weight air dry, gm		3 2	16 2
Nitrogen, gm		0 19	0 80
Nitrogen, per cent		5 93	4 91
Nitrogen utilization, per cent		95 8	82 8

TABLE 8

*Soy Bean without Agar or Bone Ash*

SUBJECT DOG 5 Weight at beginning 6.2 Kg Weight at end 6.2 Kg		PERIOD XXVI* (3 days) Soy Bean Feeding	PERIOD XXVIII (5 days) Meat Feeding
Composition of daily diet	Soy bean	grams 64	Meat 150
	Sugar	25	Sugar 25
	Lard	20	Lard 20
	Water	225	Water 100
	Estimated calories	530	Estimated calories 530
		Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm		3 66	3 68
Total nitrogen, gm		4 31	3 84
Nitrogen in food, gm		4 61	4 64
Nitrogen balance, gm		+0 30	+0 80
<i>Feces</i>			
Weight air dry, gm		18 5	3 4
Nitrogen, gm		0 65	0 16
Nitrogen, per cent		3 52	4 62
Nitrogen utilization, per cent		85 9	96 6

About half of the food forced each day

TABLE 9

*Soy Bean without Agar or Bone Ash*

SUBJECT, DOG 6 Weight at beginning 6.6 Kg Weight at end 6.6 Kg		PERIOD XXVII (3 days) Soy Bean Feeding	PERIOD XXIX (3 days) Meat Feeding
Composition of daily diet	Soy bean	64	Meat 150
	Sugar	25	Sugar 25
	Lard	20	Lard 20
	Water	225	Water 100
	Estimated calories	530	Estimated calories 530
		Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm		3.45	3.35
Total nitrogen, gm		4.06	3.59
Nitrogen in food, gm		4.61	4.64
Nitrogen balance, gm		+0.55	+1.05
<i>Feces</i>			
Weight air dry, gm		16.7	4.4
Nitrogen, gm		0.61	0.24
Nitrogen, per cent		3.64	5.42
Nitrogen utilization, per cent		86.8	94.9

TABLE 10

*Soy Bean without Agar or Bone Ash*

SUBJECT, DOG 7 Weight at beginning 6.6 Kg Weight at end, 6.6 Kg		PERIOD XXVI (4 days) Soy Bean Feeding	PERIOD XXVIII (3 days) Meat Feeding
Composition of daily diet	Soy bean	64 grams	Meat 150 grams
	Sugar	25	Sugar 25
	Lard	20	Lard 20
	Water	225	Water 100
	Estimated calories	530	Estimated calories 530
		Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm		3.78	3.60
Total nitrogen, gm		4.35	3.75
Nitrogen in food, gm		4.61	4.64
Nitrogen balance, gm		+0.26	+0.89
<i>Feces</i>			
Weight air dry, gm		15.9	3.8
Nitrogen, gm		0.57	0.15
Nitrogen, per cent		3.60	3.82
Nitrogen utilization, per cent		87.6	96.9

TABLE 11

*Soy Bean without Agar or Bone Ash*

SUBJECT DOG 5 Weight at beginning 6.0 Kg Weight at end 5.9 Kg	PERIOD XVI (4 days) Meat Feeding	PERIOD XVII (4 days) Soy Bean Feeding
Composition of daily diet	Meat <sup>grams</sup> 100	Soy bean <sup>grams</sup> 46
	Sugar 25	Sugar 25
	Lard 20	Lard 20
	Water 150	Water 225
	Estimated calories 450	Estimated calories 460
Daily Averages		Daily Averages
Nitrogen output		
Urine nitrogen, gm	2.77	2.83
Total nitrogen, gm	2.79	3.48
Nitrogen in food, gm	3.28	3.31
Nitrogen balance, gm	+0.49	-0.17
Feces		
Weight air dry, gm	0.4	14.0
Nitrogen, gm	0.02	0.65
Nitrogen, per cent	5.32	4.67
Nitrogen utilization, per cent	99.4*	80.2

Feces evidently separated imperfectly

TABLE 12

*Soy Bean without Agar or Bone Ash*

SUBJECT DOG 6 Weight at beginning 6.2 Kg Weight at end 6.1 Kg	PERIOD XVII (4 days) Meat Feeding	PERIOD XVIII (4 days) Soy Bean Feeding
Composition of daily diet	Meat <sup>grams</sup> 100	Soy bean <sup>grams</sup> 46
	Sugar 25	Sugar 25
	Lard 20	Lard 20
	Water 150	Water 225
	Estimated calories 450	Estimated calories 460
Daily Averages		Daily Averages
Nitrogen output		
Urine nitrogen, gm	2.41	2.49
Total nitrogen, gm	2.54	3.21
Nitrogen in food, gm	3.28	3.31
Nitrogen balance, gm	+0.74	+0.10
Feces		
Weight air dry, gm	1.9	14.0
Nitrogen, gm	0.13	0.72
Nitrogen, per cent	7.03	5.15
Nitrogen utilization, per cent	96.0	79.3

TABLE 13

*Soy Bean without Agar or Bone Ash*

SUBJECT DOG 7 Weight at beginning 6.0 Kg Weight at end 5.0 Kg		PERIOD XVI (4 days) Meat Feeding	PERIOD XVII (4 days) Soy Bean Feeding
Composition of daily diet		<i>grams</i>	<i>grams</i>
	Meat	100	Soy bean 46
	Sugar	25	Sugar 25
	Lard	20	Lard 20
	Water	150	Water 225
	Estimated calories	450	Estimated calories 460
	Daily Averages		Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm		2.85	2.83
Total nitrogen, gm		2.95	3.37
Nitrogen in food, gm		3.28	3.31
Nitrogen balance, gm		+0.33	-0.06
<i>Feces</i>			
Weight air dry, gm		1.5	11.0
Nitrogen, gm		0.10	0.54
Nitrogen, per cent		7.06	4.77
Nitrogen utilization, per cent		96.8	83.8

WHITE BEAN *Man, Table 14* The original intention was to investigate the digestibility of hulled beans which had been finely ground and thoroughly cooked, and in which the starch had in great part been dissolved by an amylolytic preparation. This mixture, however, produced such violent nausea that a successful experiment with it was entirely out of the question. Indeed other workers, notably Strumpell, have reported similar difficulties with bean experiments.

However, the subject was unwilling to have the fore period stand for naught, so the experiment was carried through using ordinary unhulled beans, which were cooked or baked in the usual way. In this form the beans were far from unpalatable. The character of the dietary employed in this experiment is given below.

	PRELIMINARY AND FORE PERIODS	EXPERIMENTAL PERIOD
	Daily Averages	Daily Averages
	grams	grams
Cracker	70	35
Egg	200	90
Meat	200	
Beans		230
Potato	100	
Tomato	250	300
Apple	200	200
Orange	180	180
Grapes	120	140
Milk	60	60
Sugar	120	130
Butter	75	80
Cereal coffee, tea	600	600

TABLE 14

## White Bean

SUBJECT MAN Weight at beginning 56.4 Kg Weight at end 56.0	PERIOD I (4 days) Mixed Diet	PERIOD II (4 days) White Bean
Composition of daily diet	Meat, egg, potato, fruit, etc	Beans, egg, fruit, etc 68.2 per cent of total nitrogen supplied by the beans
	Estimated calories 2600	Estimated calories 2700
Nitrogen output	Daily Averages	Daily Averages
Urine nitrogen, gm	9.19	8.00
Total nitrogen, gm	10.90	10.70
Nitrogen in food, gm	12.25	12.20
Nitrogen balance, gm	-1.35	-1.50
Feces		
Weight, air dry, gm	26.2	44.2
Nitrogen, gm	1.71	2.70
Nitrogen, per cent	6.54	6.11
Nitrogen utilization, per cent	86.0	77.9

The plan of the experiment differed in no essential from those on man already reported. The preliminary period of adjustment was only one day in duration, and the after period was entirely

omitted. The results are in general accord with those obtained by previous observers.

The factors which probably contribute to the unfavorable utilization of the protein of beans have already been discussed.<sup>1</sup> The plan was conceived of avoiding these unfavorable influences as far as possible, and to that end, as already described,<sup>2</sup> hulled and powdered beans were thoroughly cooked, and the greater part of the starch removed. Experiments with this material follow.

**CRUDE BEAN PROTEIN<sup>3</sup>—Dogs, Tables 15 to 21** The material was not dried, but when in the stage of evaporation the proportion of solid matter became about 20 per cent, it was pressed into packages containing the daily supply of protein, and preserved frozen until ready for use. In some cases, no additional water was given, as the moisture of the product sufficed. Such details may be most readily learned from the tables.

In the experiments reported in Tables 15 to 17, the food mixture was of the consistency of putty and made a rather large volume of material. The animals experienced some difficulty in chewing because of the unusual consistency, but in spite of this Dogs 6 and 7 appeared to relish the fare, and even Dog 5, upon whom forced feeding had to be practiced, did not seem to find the food especially repellent.

It was thought that possibly this large mass of food overburdened the digestive tract thus accounting for the rather poor utilization, and hence experiments reported in Tables 18 to 20 were instituted, where the food nitrogen was only two-thirds as great as in the preceding three experiments.

In all the above experiments, agar and bone ash were included in the diet, and it was therefore desired to eliminate the influence of these "indigestibles" for comparison. Hence the experiment recorded in Table 21.

<sup>1</sup> Cf. Mendel and Fine. *This Journal*, v, p. 305, 1911.

<sup>2</sup> Pp. 435-436.

<sup>3</sup> As far as we are aware the only experiments with similar material were conducted by Edsall and Miller (see bibliography) on infants and on a man. The infants digested 90 per cent or over of the nitrogen in the bean periods and the man utilized 94 per cent. However, the bean protein furnished but 25 per cent of that of the infant's food and only about 12 per cent of the protein in the man's dietary, whereas in our experiments all the protein was supplied by the bean preparation.

TABLE 15  
*Crude Bean Protein with Agar and Bone Ash*

SUBJECT DOG 5 Weight at beginning 6.5 Kg Weight at end 6.2 Kg		PERIOD VI (4 days) Meat Feeding	PERIOD VII* (3 days) Bean Protein Feeding
Composition of daily diet		grams	grams
	Meat	150	Bean protein 300
	Sugar	20	Sugar 20
	Lard	20	Lard 25
	Agar	2	Agar 2
	Bone ash	5	Bone ash 5
	Water	100	Water (contain- ed in the bean protein) 240
	Estimated calories	520	Estimated calories, 560
Nitrogen output		Daily Averages	Daily Averages
Urine nitrogen, gm		4 65	4 24
Total nitrogen, gm		4 97	5 08
Nitrogen in food, gm		5 23	5 10
Nitrogen balance, gm		+0 26	+0 02
Feces			
Weight air dry, gm		11 0	39 3
Nitrogen, gm		0 32	0 84
Nitrogen, per cent		2 95	2 15
Nitrogen utilization, per cent		93 8	83 4

Forced feeding necessary throughout the period

TABLE 16  
*Crude Bean Protein with Agar and Bone Ash*

SUBJECT DOG 6 Weight at beginning 7.1 Kg Weight at end 6.8 Kg		PERIOD VI (4 days) Meat Feeding	PERIOD VII (3 days) Bean Protein Feeding
Composition of daily diet		grams	grams
	Meat	150	Bean protein 300
	Sugar	20	Sugar 20
	Lard	20	Lard 25
	Agar	2	Agar 2
	Bone ash	5	Bone ash 5
	Water	100	Water (contain- ed in the bean protein) 240
	Estimated calories	520	Estimated calories 560
Nitrogen output		Daily Averages	Daily Averages
Urine nitrogen, gm		3 84	3 62
Total nitrogen, gm		4 20	4 59
Nitrogen in food, gm		5 23	5 10
Nitrogen balance, gm		+1 03	+0 51
Feces			
Weight air dry, gm		9 2	35 3
Nitrogen, gm		0 36	0 97
Nitrogen, per cent		3 91	2 75
Nitrogen utilization, per cent		93 1	81 0



TABLE 17  
*Crude Bean Protein with Agar and Bone Ash*

SUBJECT, DOG 7 Weight at beginning, 6.7 Kg Weight at end, 6.4 Kg		PERIOD X (4 days) Meat Feeding	PERIOD XI (3 days) Bean Protein Feeding
Composition of daily diet		grams	grams
	Meat	150	Bean protein 300
	Sugar	20	Sugar 20
	Lard	20	Lard 25
	Agar	2	Agar 2
	Bone ash	5	Bone ash 5
	Water	100	Water (contained in the bean protein) 240
	Estimated calories	520	Estimated calories 560
<i>Nitrogen output</i>		Daily Averages	Daily Averages
Urine nitrogen, gm		3.84	3.51
Total nitrogen, gm		4.16	4.37
Nitrogen in food, gm		5.23	5.10
Nitrogen balance, gm		+1.07	+0.73
<i>Feces</i>			
Weight air dry, gm		10.0	36.3
Nitrogen, gm		0.32	0.86
Nitrogen, per cent		3.20	2.38
Nitrogen utilization, per cent		93.9	83.1

TABLE 18  
*Crude Bean Protein with Agar and Bone Ash*

SUBJECT, DOG 5 Weight at beginning 6.2 Kg Weight at end, 5.9 Kg		PERIOD XII (4 days) Meat Feeding	PERIOD XIII* (4 days) Bean Protein Feeding	PERIOD XIV (4 days) Meat Feeding
Composition of daily diet		grams	grams	grams
	Meat	100	Bean protein 200	Meat 100
	Sugar	25	Sugar 25	Sugar 25
	Lard	20	Lard 25	Lard 25
	Agar	2	Agar 2	Agar 2
	Bone ash	5	Bone ash 5	Bone ash 5
	Water	150	Water (160 gm in the bean protein) 225	Water 150
	Estimated calories	450	Estimated calories 480	Estimated calories 450
<i>Nitrogen output</i>		Daily Averages	Daily Averages	Daily Averages
Urine nitrogen, gm		3.15	3.00	2.64
Total nitrogen, gm		3.43	3.54	2.90
Nitrogen in food, gm		3.49	3.34	3.28
Nitrogen balance, gm		+0.06	-0.20	+0.38
<i>Feces</i>				
Weight air dry, gm		11.0	26.5	11.5
Nitrogen, gm		0.28	0.54	0.26
Nitrogen, per cent		2.59	2.05	2.27
Nitrogen utilization, per cent		91.8	83.8	92.1

\* Forced feeding necessary throughout the period

TABLE 19  
*Crude Bean Protein with Agar and Bone Ash*

SUBJECT DOG 6 Weight at beginning 6.6 Kg Weight at end 6.3 Kg		PERIOD XIII (4 days) Meat Feeding	PERIOD XIV (4 days) Bean Protein Feeding	PERIOD XV (4 days) Meat Feeding
Composition of daily diet		Meat <i>grams</i> 100	Bean pro- tein <i>grams</i> 200	Meat <i>grams</i> 100
		Sugar 25	Sugar 25	Sugar 25
		Lard 20	Lard 25	Lard 20
		Agar 2	Agar 2	Agar 2
		Bone ash 5	Bone ash 5	Bone ash 5
		Water 150	Water (160 gm in the bean protein) 225	Water 150
		Estimated calories 450	Estimated calories 480	Estimated calories 450
<i>Nitrogen output</i>		Daily Averages	Daily Averages	Daily Averages
Urine nitrogen, gm		2 56	2 64	2 40
Total nitrogen, gm		2 91	3 33	2 71
Nitrogen in food, gm		3 49	3 34	3 28
Nitrogen balance, gm		+0 58	+0 01	+0 57
<i>Feces</i>				
Weight air dry, gm		10 5	28 3	10 0
Nitrogen, gm		0 35	0 69	0 31
Nitrogen, per cent		3 37	2 45	3 07
Nitrogen utilization, per cent		89 8	79 3	90 6

TABLE 20  
*Crude Bean Protein with Agar and Bone Ash*

SUBJECT DOG 7 Weight at beginning 6.4 Kg Weight at end 6.1 Kg		PERIOD XII (4 days) Meat Feeding	PERIOD XIII (4 days) Bean Protein Feeding	PERIOD XIV (4 days) Meat Feeding
Composition of daily diet		Meat <i>grams</i> 100	Bean pro- tein <i>grams</i> 200	Meat <i>grams</i> 100
		Sugar 25	Sugar 25	Sugar 25
		Lard 20	Lard 25	Lard 20
		Agar 2	Agar 2	Agar 2
		Bone ash 5	Bone ash 5	Bone ash 5
		Water 150	Water (160 gm in the bean protein) 225	Water 150
		Estimated calories 450	Estimated calories 480	Estimated calories 450
<i>Nitrogen output</i>		Daily Averages	Daily Averages	Daily Averages
Urine nitrogen, gm		2 82	2 70	2 55
Total nitrogen, gm		3 05	3 34	2 81
Nitrogen in food, gm		3 49	3 34	3 28
Nitrogen balance, gm		+0 44	±0 00	+0 47
<i>Feces</i>				
Weight air dry, gm		8 5	25 3	9 5
Nitrogen, gm		0 23	0 64	0 26
Nitrogen, per cent		2 74	2 52	2 70
Nitrogen utilization, per cent		93 3	80 9	92 2

TABLE 21  
*Crude Bean Protein without Agar or Bone Ash*

SUBJECT DOG 7 Weight at beginning 6.0 Kg Weight at end 5.9 Kg		PERIOD XVI (4 days) Meat Feeding	PERIOD XVII (3 days) Bean Protein Feeding
Composition of daily diet		<i>grams</i>	<i>grams</i>
	Meat	100	Bean protein 235
	Sugar	25	Sugar 25
	Lard	20	Lard 20
	Water	150	Water (180 gm in the bean protein) 230
	Estimated calories	450	Estimated calories 480
<i>Nitrogen output</i>		Daily Averages	Daily Averages
Urine nitrogen, gm		2.85	2.58
Total nitrogen, gm		2.95	3.21
Nitrogen in food, gm		3.28	3.43
Nitrogen balance, gm		+0.33	+0.22
<i>Feces</i>			
Weight air dry, gm		1.5	24.6
Nitrogen, gm		0.10	0.63
Nitrogen, per cent		7.06	2.57
Nitrogen utilization, per cent		96.8	81.5

One would at once gain the impression from these results that the legume proteins are among the less well utilized materials, nor is this view dispelled when we attempt to analyze certain possible contributing causes. The bean diets contained celluloses and hemicelluloses, substances inherent in the experimental material and entirely or for the most part indigestible. The influence upon utilization of such non-nitrogenous matter cannot be ascertained from the foregoing tables. In a few instances the attempt has been made to determine the amounts of these substances which have failed to disappear from the alimentary tract. The crude fiber of the food may be assumed to completely reappear in the feces<sup>1</sup>. The undigested hemicelluloses of the excrement was determined by the method outlined in a previous paper<sup>2</sup>. These data are presented in Table 22 and are in part reproduced in Table

<sup>1</sup> Cf Swartz *Transactions of the Connecticut Academy of Arts and Sciences*, xvi, p 268, 1911

<sup>2</sup> Mendel and Fine *This Journal*, v, p 339, 1911

23, where the utilization of the legume proteins is compared to that of the protein of meat diets containing comparable or greater amounts of indigestible non-nitrogenous matter. From the latter table it is apparent that the presence of indigestible non-nitrogenous matter is not wholly responsible for the low coefficients of digestibility of the proteins of the soy bean and crude bean preparations, since the coefficients for the nitrogen of meat diets including like or greater amounts of such substances was distinctly higher. This is further borne out in the following two experiments (Tables 24 and 25) with isolated legume proteins.

TABLE 22

*Undigested Carbohydrates Derived from the Food Material Daily Values*

TABLE	MATERIAL UNDER INVESTIGATION	WEIGHT OF INGESTED LEGUME (DRY)	COMPOSITION OF THE FECES		
			Crude Fiber	Hemicellulose	Total
		grams	grams	grams	grams
11	Soy Bean	46	1 0	3 7	4 7
12	Soy Bean	46	1 0	3 5	4 5
13	Soy Bean	46	1 0	2 9	3 9
5	Soy Bean	64	1 5	5 4	6 9
6	Soy Bean	64	1 5	5 2	6 7
21	Crude Bean Protein	40	4 4	9 8	14 2

Cf. the analyses on pp. 433 and 436

TABLE 23

*Comparison of the Utilization of Proteins in Relation to the Content of Indigestible Non-Nitrogenous Materials in the Diet Daily Averages*

TABLE	NATURE OF INGESTA	NITROGEN INTAKE	INDIGESTIBLE NON-NITROGENOUS MATTER OF THE DIET	NITROGEN UTILIZATION
		grams	grams	per cent
11	Soy Bean	3 3	4 7	80 2
12	Soy Bean	3 3	4 5	79 3
13	Soy Bean	3 3	3 9	83 8
5	Soy Bean	4 6	6 9	85 0
6	Soy Bean	4 6	6 7	84 0
21	Crude Bean Protein	3 2	14 2	81 5
	Meat*	3 3	13 0	89 2
	Meat*	3 3	6 0	91 0

\*These data will be discussed more fully in a subsequent paper of this series. In each case the average of three experiments is presented.

TABLE 24

*Phaseolin with Agar and "Salts"\**

SUBJECT DOG 4 Weight at beginning 5.1 Kg Weight at end, 5.1 Kg		PERIOD IX (3 days) Meat Feeding	PERIOD X (5 days) Phaseolin Feeding	PERIOD XI (5 days) Meat Feeding
Composition of daily diet	<i>grams</i>		<i>grams</i>	<i>grams</i>
	Meat	150	Phaseolin	38
	Sugar	25	Sugar	25
	Starch	5	Starch	5
	Lard	20	Lard	30
	Agar	8	Agar	8
	Salts	4	Salts	4
	Water	200	Water	300
	Estimated calories	540	Estimated calories	520
				Estimated calories 540
	Daily Averages		Daily Averages	Daily Averages
<i>Nitrogen output</i>				
Urine nitrogen, gm		4 45	4 11	4 28
Total nitrogen, gm		4 63	5 32	4 62
Nitrogen in food, gm		5 27	5 22	5 20
Nitrogen balance, gm		+0 64	-0 10	+0 58
<i>Feces</i>				
Weight air dry, gm		9 4	20 0	12 4
Nitrogen, gm		0 18	1 21	0 34
Nitrogen, per cent		1 93	6 07	2 77
Nitrogen utilization, per cent		96 5	76 9	93 4

\*The ingredients of this salt mixture were reported by Mendel and Fine. *This Journal* x, p 321, 1911

**PHASEOLIN** *Dog, Table 24* The food mixture, including water, as detailed in the experimental period reported in Table 24, was heated on a water bath for four to six hours. The cooking of the food mixture resulted in the gathering together of the material in small lumps, which may possibly account for the *very unfavorable utilization*. We do not believe that this condition appreciably influenced the result, since the lumps were not hard and solid, but on the contrary, quite pervious. Repeated attempts to feed this preparation in food mixtures, *not* subjected to heat, invariably resulted in complete failure. Ingestion of the material was regularly followed by nausea and vomiting.

**PEA GLOBULIN** *Dog, Table 25* The pea globulin as used in this experiment was entirely soluble in 10 per cent sodium chloride and was an ideal material for the study of the problem in hand.

The food mixture was not heated as in the preceding experiment, but in spite of this the material gathered together in small pervious lumps, when the water was added. Although the *utilization does not fall far below that of meat*, fed under similar conditions, yet in the relatively high nitrogen content of the feces we note the evidence of the escape of a portion of the protein from digestion.

As far as we are aware, Salkowski is the only other worker who has studied the utilization of the isolated legume protein. This author found the isolated protein of the horse bean to be 94 per cent utilized, against a utilization of 89 per cent for the untreated horse bean. It should be noted that Salkowski's protein was prepared by extracting the beans with dilute alkali and precipitating the dissolved protein with acid.

TABLE 2a  
*Pea Globulin with Agar and Bone Ash*

SUBJECT DOG 6 Weight at beginning 49 Kg Weight at end 46 Kg		PERIOD VI (4 days) Meat Feeding	PERIOD VII (5 days) Pea Globulin Feeding	PERIOD VIII (5 days) Meat Feeding
Composition of daily diet	Meat	grams 150	Pea Glob- ulin 30	Meat 150
	Sugar	20	Sugar 20	Sugar 20
	Lard	20	Lard 30	Lard 20
	Agar	3	Agar 3	Agar 3
	Bone Ash	7	Bone Ash 7	Bone Ash 7
	Water	100	Water 200	Water 100
	Estimated calories	520	Estimated calories 470	Estimated calories 520
<i>Nitrogen output</i>		Daily Averages	Daily Averages	Daily Averages
Urine nitrogen, gm		4 13	5 14	4 23
Total nitrogen, gm		4 49	5 70	4 62
Nitrogen, in food, gm		4 93	4 81	4 80
Nitrogen balance, gm		+0 45	-0 89	+0 18
<i>Feces</i>				
Weight air dry, gm		14 5	15 6	15 0
Nitrogen, gm		0 35	0 56	0 39
Nitrogen, per cent		2 42	3 62	2 59
Nitrogen utilization, per cent		92 9	88 3	91 9

Although the data reported in this paper would seem to warrant the conclusion that the leguminous proteins are relatively poorly

utilized, this must be accepted with some reservation, at least until the influence of varying quantities of certain indigestible materials upon the utilization of easily digested substances like meat can be ascertained, and a larger number of trials with the isolated proteins are carried out

TABLE 26  
*Nitrogen Balances in Soy Bean Experiments*

SUBJECT	TABLE	SOY BEAN	MEAT
Man	1	+0 21	+1 67, +1 06
Dog 1	2	-0 50	-0 22, +1 06
Dog 5	3	+0 64	+0 82, +0 36
Dog 7	4	+0 30	+0 40, +0 41
Dog 5	5	-0 05	+0 29
Dog 6	6	+0 45	+0 82
Dog 7	7	+0 40	+0 85
Dog 5	8	+0 30	+0 80
Dog 6	9	+0 55	+1 05
Dog 7	10	+0 26	+0 89
Dog 5	11	-0 17	+0 49
Dog 6	12	+0 10	+0 74
Dog 7	13	-0 06	+0 33

TABLE 27  
*Nitrogen Balances in Crude Bean Experiments*

SUBJECT	TABLE	CRUDE BEAN PROTEIN	MEAT
Dog 5	15	+0 02	+0 26
Dog 6	16	+0 51	+1 03
Dog 7	17	+0 73	+1 07
Dog 5	18	-0 20	+0 06, +0 38
Dog 6	19	+0 01	+0 58, +0 57
Dog 7	20	+0 00	+0 44, +0 47
Dog 7	21	+0 22	+0 33

TABLE 28  
*Nitrogen Balances in Experiments on Phaseolin and Pea Globulin*

SUBJECT	TABLE	PHASEOLIN	PEA GLOBULIN	MEAT
Dog 4	24	-0 10		+0 64, +0 58
Dog 6	25		-0 89	+0 45, +0 18

In Tables 26 to 28 are collected the nitrogen balances obtained in the experiments reported in the present paper. The soy bean and crude bean proteins maintained generous positive balances which are, however, uniformly smaller than those for meat. This difference is explained by the increased elimination of fecal nitrogen during the bean periods. A similar explanation accounts for the negative balance during the phaseolin period, but the unfavorable nitrogen balance during the feeding of pea globulin is presumably attributable to its inherent inadequacy.

#### SUMMARY

In comparison with the other vegetable proteins thus far reported in this series of studies, the legume proteins are less well utilized. The materials investigated principally were (1) soy bean flour, free from starch, (2) a product prepared from the white bean by thoroughly disintegrating the cells and dissolving and washing out the starch, (3) phaseolin—a protein isolated from the white bean, and (4) an uncoagulated globulin from the garden pea. The unfavorable results with the soy bean and white bean preparations can be explained only in part by the presence of cellulose and hemicellulose in these products. Such considerations cannot be applied to the data for phaseolin and pea globulin.

Attention was called to the desirability of further work on the isolated legume proteins, and on the influence of indigestible non-nitrogenous materials upon the utilization of meat.

The observations regarding the soy bean are of special interest in view of the fact that this product has lately been introduced quite widely as an adjuvant to the dietary of diabetics.

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# HOW DO ISOTONIC SODIUM CHLORIDE SOLUTION AND OTHER PARTHENOGENIC AGENTS INCREASE OXIDATION IN THE SEA URCHIN'S EGG?

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(Received for publication, November 4, 1911 )

Loeb has shown that  $\text{OH}^-$  ions favor development<sup>1</sup> Our own experiments (Table 6) as well as those of O Warburg<sup>2</sup> demonstrate that increase in the alkalinity of the medium increases oxidation in fertilized eggs

According to Warburg, the egg is impermeable to  $\text{OH}^-$  ions or fixed alkalis, because, although eggs stained with neutral red are changed to yellow by  $\text{NH}_3$  of a concentration which increases the oxidation only one-tenth, similar eggs are not changed to yellow by fixed alkalis of a concentration at which oxidation is greatly increased

These facts are, however, capable of another interpretation The egg is filled with lipid particles which take up the neutral red to such an extent as to render the surrounding protoplasm and sea water colorless The  $\text{OH}^-$  ions cannot freely enter the lipoids in order to change the color of the neutral red On the other hand ammonia is lipid-soluble and can enter

It might be objected that the ammonia cannot react with the dye in the non-aqueous medium owing to the suppression of ionization, but whether the dye is driven out of the lipoids or changed to yellow *in situ*, the fact remains that it does become yellow

Harvey<sup>3</sup> observed that the addition of but a small quantity of alkali to sea water containing fertilized eggs stained with neutral

<sup>1</sup> Loeb *Chemische Entwicklungserregung des tierischen Eies* Berlin, 1909

<sup>2</sup> Warburg *Zeitschr f physiol Chem*, lx, p 305, 1910

<sup>3</sup> Harvey *Journ of Exp Zoology*, v, p 507, 1911

red, changed the eggs to yellow. However, the eggs were injured by the alkali and probably their permeability was increased. It is possible that alkalies or  $\text{OH}^-$  ions in any concentration enter the fertilized eggs but must be present in sufficient concentration to set free ammonia or change the lipoids in order to affect the dye.

We may assume, then, unless more conclusive evidence indicates the contrary, that the  $\text{OH}^-$  ions increase oxidation after penetrating the egg.

One of us has shown that unfertilized sea urchin's eggs are poorly permeable to salts and their ions, but become more permeable after fertilization or the initiation of parthenogenetic development.<sup>1</sup> Not only is the permeability increased but the oxidation rate is increased. Warburg observed that oxidation increases from five to seven times on fertilization (compare our Table 5, experiment III). He found also a large increase after the initiation of parthenogenetic development caused by hypertonic sea water,<sup>2</sup> fatty acid, alkalies or traces of the heavy metals,<sup>3</sup> silver or copper. Our own experiments, given below, confirm and extend these findings. There appears then to be some relation between permeability and oxidation, and the present paper is an attempt to determine what this relation is.

The living cell may be compared to a furnace, and R. Lillie<sup>4</sup> advanced the view that increase in permeability opens the draughts so to speak, allowing the escape of carbonic acid, and hence oxidation is increased. He supposes that the accumulation of carbonic acid and perhaps other end-products checks the oxidation, and increase in permeability to carbonic acid allows oxidation to proceed. The difficulty with this hypothesis lies in the fact that living cells have been shown by Overton and others to be freely permeable to substances which are easily soluble in fats and oils, or especially in lecithin and cholesterol. Carbon dioxide is soluble in oils and probably enters cells easily, at least there is evidence to show that red blood corpuscles are freely permeable to this gas. Fatty oils are permeable only to the undissociated molecules and not to the ions. Since the proportion of ions of carbonic

<sup>1</sup> McClendon *Amer Journ of Physiol*, LVII, p 240, 1910

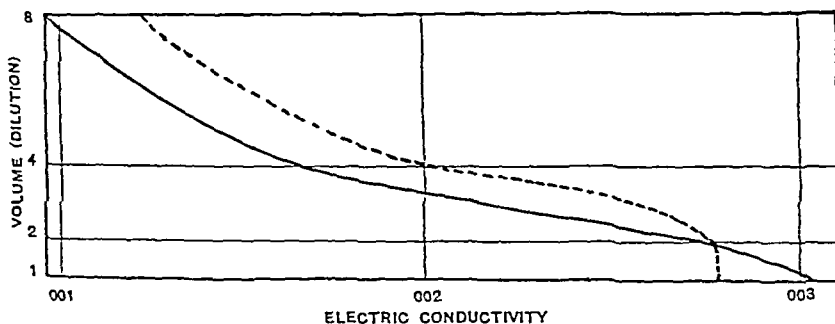
<sup>2</sup> Warburg *Zeitschr f physiol Chem*, LVII, p 1, 1908

<sup>3</sup> Warburg *Ibid*, LVII, p 305, 1910

<sup>4</sup> Lillie *Biol Bull*, LVII, p 188, 1909

acid would ordinarily be small, what conditions in the egg might favor ionization of  $\text{CO}_2$ ?

Not all of the alkali metals in the egg are combined with mineral acids, some are combined with proteins. This has been shown by the senior author with electric conductivity measurements of hens egg yolk given in the accompanying curves. The continuous line represents the conductivity of yolk freed from protein granules, and the dotted line, yolk containing an excess of protein



granules, separated by the centrifuge. The granules impede the current as shown by the fact that the granule-containing yolk is a poorer conductor than granule-free yolk. But on dilution, the granule-containing yolk becomes the better conductor. Therefore ions are set free from the granules on dilution.

The carbonic acid formed within the egg would react with the alkali albuminates with the formation of alkali carbonates and bicarbonates, which, notwithstanding hydrolysis, would liberate a considerable quantity of carbonic acid anions<sup>1</sup>. The dissociated carbonic acid, being unable to escape from the unfertilized egg, would lower the  $\text{OH}^-$  ion concentration and thus reduce oxidation. As the undissociated molecules of carbonic acid escape, more are formed by the slow oxidation in the egg.

On fertilization, the permeability to the anions of carbonic acid is greatly increased. They migrate out of the egg, and negative ions enter the egg to take their place. Since the  $\text{OH}^-$  ions of the sea water are the fastest negative ions, they enter the egg and

<sup>1</sup> At about molecular concentration the equivalent electric conductivity of  $\text{NaCl}$  is 76, of  $\text{Na}_2\text{CO}_3$  45 at  $18^\circ$ .

increase oxidation. However, if some  $\text{-Cl}$  or  $\text{-SO}_4$  ions entered the egg they would tend to decrease the  $\text{-OH}$  ion concentration within the egg. But the senior author has shown that the fertilized *Fundulus* egg is impermeable to  $\text{-Cl}$  ions, for when placed in distilled water, or in solutions of nitrates or sulphates, practically no chlorine comes out of the eggs<sup>1</sup>. We may assume, therefore, that carbonic acid accumulates in the unfertilized egg until the reaction is neutral or slightly acid. But on fertilization, the permeability to carbonic acid anions is increased and the concentration of this acid is diminished so that the reaction is neutral or slightly alkaline. The increased alkalinity is the cause of the increased oxidation. We will now see to what extent our experiments bear out this assumption.

We observed that oxidation is about doubled when the egg is made parthenogenetic with carbonated sea water (Table 5, experiment II) or alkaline isotonic sodium chloride (Table 3, experiments I and II). In some cases the eggs, being physiologically different from those in other experiments, did not show the morphological signs of development in this solution, and oxidation was not doubled (Table 2, Table 3, experiment III, cf. Table 5, experiment I). The eggs begin development while in the alkaline solution, but eggs made parthenogenetic by treatment with neutral or acid solutions (neutral sodium chloride or carbonated sea water) begin development only when transferred to natural sea water or other alkaline solution. This bears out our hypothesis, for if the increased permeability remains after the egg is returned to an alkaline medium, a chance is given for an increase in alkalinity in the interior, which was lacking in the non-alkaline solution.

Certain facts may seem to contradict our assumption, but probably they merely limit its application.

1. A slight increase in  $\text{-OH}$  ions may cause even the unfertilized egg to absorb more oxygen (Table 2) and a greater increase causes it to develop. This does not necessarily show permeability of the unfertilized egg to hydroxyl ions. The increased alkalinity slowly causes an increased permeability of the egg and thus leads to parthenogenesis, but the degree of alkalinity of the medium necessary to induce development of the unfertilized egg is far greater

<sup>1</sup> These experiments will be published later in the *American Journal of Physiology*.

than that necessary for the development of the fertilized egg or the egg already made parthenogenetic

2 The fertilized egg of *Arbacia punctulata* (but not of some other sea urchins) may develop in a natural medium, as Loeb observed and which we have confirmed. In other words, a hydroxyl ion concentration in the medium, greater than that of distilled water, is not necessary for development of this egg made freely permeable to carbonic acid. However this fact does not set a limit to the alkalinity of the egg interior. The egg probably contains more Na than Cl ions, and if it be impermeable to Na or Cl, the escape of carbonic acid might cause the egg interior to become alkaline or at least neutral. Eggs made parthenogenetic in some ways (neutral sodium chloride, for instance) do not develop unless transferred to an alkaline medium, but this may be due to the possibility that these parthenogenetic eggs are not quite as permeable as are fertilized eggs. The same may be inferred from oxidation measurements. Neutral sodium chloride causes the unfertilized egg to absorb more oxygen than it does in sea water (Table 4) but the increase is slight, and morphological development does not commence. If the eggs are then transferred to sea water or other alkaline solution, some of them may develop.

It appears therefore that increase in permeability is a gradual process. Although some eggs are so permeable as to be able to develop in an neutral medium others are less permeable and do not develop, or develop only in an alkaline medium. By treating eggs with parthenogenetic agents in various concentrations or for various lengths of time we may induce various degrees of permeability. Even fertilized eggs may be made more permeable by treatment with parthenogenic agents, and a corresponding increase in oxidation may be observed (Table 6). In these experiments the oxidation of the eggs in sea water was measured about ninety minutes after fertilization. They were then placed in isotonic, alkaline sodium chloride solution, in which the oxidation increased one-half, when returned to sea water the oxidation fell below its previous level in the same medium. According to Loeb, this indicates death of some of the eggs (20 per cent).

The experiments just described explain the discrepancy between the results of Warburg and those of Loeb. Warburg<sup>1</sup> found that

<sup>1</sup> Warburg *loc cit*

the oxidation of the *fertilized egg* in isotonic sodium chloride solutions containing a trace of sodium cyanide, is much greater than in sea water containing the same concentration of sodium cyanide. Loeb<sup>1</sup> confirmed this determination, but observed further that if the cyanide is omitted (from both) no increased oxidation in sodium chloride solution occurs. The cyanogen in both sea water and sodium chloride solution depresses oxidation. Since sodium cyanide liberates  $\text{-OH}$  ions, we may conclude that the increase in oxidation in the sodium chloride solution used by Warburg was due to the increased penetration of hydroxyl ions, following increase of permeability.

In our experiments no cyanide was used, and the alkalinity of the sodium chloride solution was not greater than sea water, yet oxidation was increased. In Loeb's experiment the tendency of increased permeability to increase oxidation was counteracted by the effect of lower alkalinity, which decreases oxidations.

Alkaline sodium chloride solution also favors oxidation in eggs that have reached later stages of development, morula or blastula (Table 7). In this experiment, the rate of oxidation in sea water was rising gradually (see next section) before the eggs were placed in the alkaline sodium chloride solution, but in the latter a sudden increase of more than 50 per cent was observed.

#### MATERIALS AND METHODS

The eggs of the sea urchins, *Arbacia punctulata*, were used. The animals were washed in a strong stream of fresh water and opened with precautions against introducing spermatozoa among the eggs. The ovaries were removed and placed in the first solution to be used, sea water or neutral van't Hoff's solution. The mass was strained through bolting cloth of such a grade as to allow but one egg to pass through one mesh at a time. The eggs were repeatedly precipitated by gravity in fresh portions of the solution in order to remove coelomic fluid cells (elaeocytes), and transferred with a small quantity of fluid to the determination flask.

The rate of oxidation in the various solutions was measured by comparison of the dissolved oxygen in the solution before and after the eggs had been suspended in it during a definite period.

<sup>1</sup> Loeb and Wasteneys *Biochem Zeitschr*, LVIII, p 340, 1910

Winkler's thiosulphate method of oxygen determination (iodometric), as described in Treadwell's Quantitative Analysis, was used

From 3 to 7 cc of eggs were used in each experiment, but the actual volume of the eggs was not measured until after the oxygen determinations

We tried a number of methods for filling the determination flask and sample bottles without an uncertain loss or gain of oxygen. Loeb collected the water in the sample bottle under petroleum. Although petroleum absorbs five times as much oxygen as water does, the oil would tend to reduce currents adjacent to the air-water surface, and thus reduce oxygen exchange. Using paraffin oil, we found that it was extremely difficult to prevent a little oil from sticking within the flask, and abandoned the method. Perhaps kerosene would have worked better, yet the quantity of kerosene that would dissolve in the water might vitiate the experiments.

Since the sea water and the solutions were shaken up and saturated with air at the given temperature before beginning the experiment, the control sample might have been taken under air, without change. But after loss of oxygen in the determination flask, a gain in oxygen would result from such treatment. By introducing the solution through a tube passing through a doubly perforated stopper, and extending to the bottom of the sample bottle, the exposed surface of the water was made as small and quiet as possible. By maintaining a constant rate of flow the error could be made to bear an approximately constant ratio to the oxidation, no matter whether the sample bottle was filled with air or some other gas. We tried the effect of introducing the sample under air, and also under hydrogen, and decided that the latter method was preferable for oxygen-low samples. In order to make all errors fall in the same direction we also collected the oxygen-high samples under hydrogen.

The experiments were so regulated that the oxygen content of the determination flask at the end of the exposure would not fall very low. However, Warburg failed to observe a decrease in the rate of oxidation in low oxygen concentration.

The water was forced out of the determination flask rapidly into the sample bottle by hydrogen under pressure. The determination



flask held 332 cc, the two sample bottles 152 and 142.6 cc respectively

The determination flask was placed in a thermostat which was kept 2° above the temperature which the air had reached at the beginning of the experiment. In most cases the time of exposure was one hour. During the first half-hour the eggs were distributed throughout the solution once every five minutes by rotating and rocking the flask, during the last half hour they were allowed to settle to the bottom.

Although the majority of the eggs settle to the bottom in ten minutes, at the end of one-half hour there are always a few which, on account of swelling or fragmentation, have failed to precipitate. To prevent these going over into the sample bottle and causing an error due to absorption of iodine, Loeb placed filter paper over the outlet. We found that a relatively hard filter paper was necessary to retain all fragments of eggs, and that this interfered with the rapid transfer of the solution. The error due to eggs in suspension is negligible, especially since it was practically constant in all of our experiments. For instance a sample bottle filled with water contained 8.09 parts per million of oxygen, while water from the same jar run into a sample bottle in which had been placed about one-hundred times as many eggs as the water from the determination flask, contained 7.85 parts per million, an error of 0.24 parts per million due to eggs in suspension. As in our experiments, differences of 0.1—3.0 parts per million were obtained, an error of 0.024 parts per million would not reverse the results.

When the eggs were fertilized or placed in parthenogenic solutions they lost sufficient red pigment (McMunn's Echinochrome) to color the water a straw yellow. In order to ascertain whether this organic matter would vitiate the results, we took two sample bottles, into one of which was placed a mass of elaeocytes containing about fifty times as much echinochrome as is lost from the eggs in one experiment, and syphoned into each tap water from the same jar. Tap water causes these cells to liberate their pigment. The bottle containing water only, was found to hold 6.85 parts per million of oxygen, while that contaminated by elaeocytes was titrated as 6.36 parts per million of oxygen. We repeated this using three sample bottles. Two of these filled with water gave

6.86 and 6.91 parts per million respectively and the third contaminated with elaeocytes liberating about one hundred times as much pigment as is liberated in the experiments with eggs, gave 6.09 parts per million as the titration, showing an error of 0.8 parts per million. Probably this loss was due chiefly to the broken up cells, but if due entirely to the pigment, the error in our experiments would be only .008 parts per million.

In experiment 3, the eggs were divided into two equal portions and placed simultaneously in two determination flasks of equal capacity. Therefore the eggs in the two solutions were in the same stage of ripeness. In each of the other experiments the eggs were all placed in one flask and treated successively with the various solutions. In case of fertilized eggs Warburg observed<sup>1</sup> that the oxidation rate rose steeply from fertilization to the 2-cell stage then gradually to the 64-cell stage. In order to determine whether this would be a great source of error on our experiments, we measured the oxygen used by a mass of fertilized eggs in sea-water during successive periods in the first six hours of development, and found it to vary from 7.93 to 9.76 tenths of a milligram (Table 7). Within the duration of the majority of our experiments, however, the variation was only from 7.93 to 8.96 tenths of a milligram or 11.5 per cent, and would be less between successive exposures. This possible source of error would not reverse the results of the majority of our experiments.

In making solutions of the same alkalinity as the sea water, a colorimetric method was used, with phenolphthalein as indicator. At the end of the season, the sea water was diluted with heavy rains and failed to color phenolphthalein. The eggs behaved abnormally, though whether this was due to the decrease in alkalinity or salinity of the sea water or to some other cause was not determined.

In making the eggs parthenogenetic with carbon dioxide, they were placed with a small quantity of sea water in a "sparklet syphon" and charged under slight pressure for about one minute. At the end of five minutes they were poured into a large volume of sea water and this was syphoned off and fresh sea water added.

<sup>1</sup> Warburg *Zeitschr f physiol Chem*, 1x, p 443, 1909 and *loc cit*

## RESULTS OF EXPERIMENTS

*I Experiments with Unfertilized Eggs*

TABLE 1

*Oxidation in neutral van't Hoff's solution contrasted with that in neutral NaCl solution*

EXPERIMENT	SOLUTION USED	VOLUME OF EGGS	TEMPERATURE THERMOSTAT	DURATION OF STIRRING	DURATION OF SETTLING	OXYGEN USED	REMARKS
		cc	°C	min	min	$\frac{mg}{10}$	
First period	{ Neutral van't Hoff }	4	24	30—	30	2 49	{ No "fertilization membrane" formed }
Second period	{ Neutral $\frac{M}{2}$ NaCl }	4	24	30	30	5 47	

TABLE 2

*Oxidation in neutral van't Hoff's solution contrasted with that in sea water and alkaline NaCl solution*

EXPERIMENT	SOLUTION USED	VOLUME OF EGGS	TEMPERATURE THERMOSTAT	DURATION OF STIRRING	DURATION OF SETTLING	OXYGEN USED	REMARKS
		cc	°C	min	min	$\frac{mg}{10}$	
First period	{ Neutral van't Hoff }		24	30	30	2 29	{ "Fertilization membranes" in very small per cent }
Second period	Sea water		24	30	30	2 65	
Third period	{ $\frac{M}{2}$ NaCl + —OH ions }		24	30	30	3 12	
Fourth period	Sea water		24	30	30	3 48	

TABLE 3

*Oxidation in sea water contrasted with that in all alicine NaCl solution*

EXPERIMENT	SOLUTION USED	VOLUME OF LUGS	TEMPERATURE THERMOSTAT	DURATION OF STIRRING	DURATION OF SETTLING	OXYGEN USED	REMARKS
		cc	°C	min	min	$\frac{mg}{10}$	
I One-half of eggs	Sea water		23	30	30	4.80	{ "Fertiliza- tion mem- brane" formed
Second half of eggs	$\left\{ \begin{array}{l} \frac{M}{2} NaCl \\ + -OH \\ ions \end{array} \right\}$		23	30	30	6.90	
II First period	Sea water		24.5	35	25	3.38	{ "Fertiliza- tion mem- brane" formed
Second period	$\left\{ \begin{array}{l} \frac{M}{2} NaCl \\ + -OH \\ ions \end{array} \right\}$		24.5	35	25	7.80	
III First period	Sea water	4.5	24	30	30	6.04	{ "Fertiliza- tion mem- branes" in very small per cent
Second period	$\left\{ \begin{array}{l} \frac{M}{2} NaCl \\ + -OH \\ ions \end{array} \right\}$	4.5	24	30	30	6.47	

TABLE 4

*Oxidation in sea water contrasted with that in neutral NaCl*

EXPERIMENT	SOLUTION USED	VOLUME OF LUGS	TEMPERATURE THERMOSTAT	DURATION OF STIRRING	DURATION OF SETTLING	OXYGEN USED	REMARKS
		cc	°C	min	min	$\frac{mg}{10}$	
I First period	Sea water	5	23	30	30	2.65	{ No "fertil- ization mem- brane" formed
Second period	$\left\{ \begin{array}{l} \text{Neutral} \\ \frac{M}{2} NaCl \end{array} \right\}$	5	23	30	30	3.60	
II First period	Sea water	2.3	23.5	30	30	1.22	Ibid
Second period	$\left\{ \begin{array}{l} \text{Neutral} \\ \frac{M}{2} NaCl \end{array} \right\}$	2.3	23.5	30	30	1.66	

TABLE 5

*Effect of CO<sub>2</sub>-parthenogenesis on oxidation      Effect of fertilization*

EXPERIMENT	SOLUTION USED	VOLUME OF EGGS	TEMPERATURE THERMOSTAT	DURATION OF STIRRING	DURATION OF SETTLING	OXYGEN USED	REMARKS
		cc	°C	min	min	$\frac{mg}{10}$	
I First period	Sea water	4.5	23.5	30	30	4.58	{ "Fertiliza- tion mem- branes" in small per cent
After CO <sub>2</sub> treatment	Sea water	4.5	23.5	30	30	6.00	
II First period	Sea water	4.5	24	30	30	4.11	{ Good mem- brane formed
After CO <sub>2</sub> treatment	Sea water	4.5	24	30	30	9.91	
III First period	Sea water		25	30	30	2.52	{ 98 per cent of eggs seg- mented
After fertili- zation	Sea water	5	25	30	30	12.94	

## SUMMARY

1 The presence of  $\text{OH}^-$  ions in the medium, increases the rate of oxidation in fertilized eggs of the sea urchin

2 The oxidation rate of unfertilized eggs is increased by fertilization or any treatment which causes them to develop parthenogenetically

In 1 and 2 we merely confirm and extend the observations of Warburg

3 Since it was shown by the senior author that fertilization or parthenogenesis means increased ionic permeability of this egg, and that the *Fundulus* egg, even after fertilization, is impermeable to  $\text{Cl}^-$  ions, the increase in permeability probably applies so far as the anions are concerned, to  $\text{OH}^-$  and  $\text{HCO}_3^-$  or  $\text{CO}_3^{2-}$  ions. The carbonic acid anions are more concentrated within, and their outward diffusion would cause a potential gradient which would pull other

## II Experiments with Fertilized Eggs

TABLE 6

*Oxidation in neutral van't Hoff's solution contrasted with that in sea water and all aine NaCl*

EXPERIMENT	SOLUTION USED	VOLUME OF LIQUID	TEMPERATURE THERMOSTAT	DURATION OF STIRRING	DURATION OF SETTling	OXYGEN USED	REMARKS
I First period	{Neutral van't Hoff}	3 6	23	30	30	6 47	{Contained some sea water
Second period	Sea water	3 6	23	30	30	7 43	
Third period	{ $\frac{M}{10}$ NaCl + $-OH$ ions}	3 6	23	30	30	12 28	
Fourth period	Sea water	3 6	23	30	30	5 97	{Very few eggs dead Eggs washed in van't Hoff sol and fertilized in it
II First period	{Neutral van't Hoff}	4	23	30	30	3 91	
Second period	Sea water	4	23	30	30	7 17	
Third period	{ $\frac{M}{10}$ NaCl + $-OH$ ions}	4	23	30	30	7 53	
Fourth period	Sea water	4	23	30	30	6 27	

anions, hence  $-OH$  ions, into the egg, thereby increasing the internal concentration of  $-OH$  ions. This increase in  $-OH$  ions probably causes the increased oxidation.

4 The increase of  $-OH$  ions in the medium causes even in unfertilized eggs, an increased oxidation. This is not interpreted as indicating that the unfertilized egg is normally permeable to  $-OH$  ions, but that increased alkalinity causes increased permeability.

5 Increase in permeability is a gradual process. Beginning with the relatively impermeable unfertilized egg, and denoting degree of permeability by numerals, we have the following series,

TABLE 7

*Effect of alkaline NaCl on oxidation six hours after fertilization*

EXPERIMENT	SOLUTION USED	VOLUME OF EGGS	TEMPERATURE THERMOSTAT	DURATION OF STIRRING	DURATION OF SETTLING	OXYGEN USED	REMARKS
		cc	°C	min	min	$\frac{mg}{10}$	
First period	Sea water	7	22	20	30	8 90	{ Thirteen minutes after fertilization
Second period	Sea water	7	22	20	30	8 96	
Third period	Sea water	7	22	20	30	8 33	2-cell stage
Fourth period	Sea water	7	22	20	30	7 93	4-cell stage
Fifth period	Sea water	7	22	20	30	8 13	16-cell stage
Sixth period	Sea water	7	22	20	30	8 13	32-cell stage
Seventh period	$\left\{ \begin{array}{l} \frac{M}{2} NaCl \\ + OH \\ ions \end{array} \right\}$	7	22	20	30	9 76	Many retarded
Eighth period	Sea water	7	22	20	30	13 77	{ Many blastulae
						8 98	All alive

I slightly increased oxidation, II greater increase in oxidation rate, and imperfect "fertilization membrane" formation, III oxidation still further increased, membrane formation perfect, followed by segmentation of the egg, IV, *ditto* except that oxidation is still further increased, and the eggs die sooner or later if oxidation is not reduced, V oxidation enormous, membrane formation but no segmentation, premature death

It is supposed that the primary effect of many toxic substances is an abnormal increase in the permeability of the egg, and fertilized eggs are more susceptible because they are already more permeable than unfertilized eggs

# CREATINE AND CREATININE METABOLISM

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(Received for publication, November 18, 1911)

In a recent number of this Journal, Mendel and his pupil Rose<sup>1</sup> have contributed a series of papers dealing with the excretion of creatinine and creatine. In this series an attempt has been made to connect the excretion of creatine in the urine with carbohydrate metabolism.

During the last six years I have been engaged in studies of normal and pathological metabolism, mainly directed to the observation of the two components of the urine which were described by Fohn as being associated with endogenous metabolism, viz, the rest nitrogen and creatinine, and at the same time I have devoted nearly equal attention to the compound so closely connected chemically with the latter, viz, creatine.<sup>2</sup>

In the course of this work, I have collected a considerable amount of information which tends to modify Mendel's views.

That under-nutrition, especially with reference to carbohydrates does induce the excretion of creatine in the urine is not questioned. The excretion of this substance is apparently not associated with deficiency in the nitrogen intake. This is shown in the results obtained by Osterberg and myself<sup>3</sup> by feeding calorically sufficient amounts of fat and carbohydrates with minimal

<sup>1</sup> Mendel and Rose. *This Journal*, x, pp 213, 240, 1911.

<sup>2</sup> Those investigations in which the excretion of creatine has been observed in pathological conditions or with coincident fasting do not properly belong to the present discussion. For completeness, they are mentioned here: Brombenzol, Marriott and Wolf. *Biochem Zeitschr*, vii, p 213, 1907, *Hydrocyanic acid*, Loewy and Wolf. *Biochem Zeitschr*, viii, p 132, 1908, *Cystinuria*, Wolf and Shaffer. *this Journal*, iv, p 439, 1908, *Carbon monoxide*, Wolf and Osterberg. *Biochem Zeitschr* xvi, 476, 1909, *Addison's Disease*, Wolf and Thacher. *Archives of Internal Medicine*, iii, p 438, 1909.

<sup>3</sup> Osterberg and Wolf. *Biochem Zeitschr*, v, p 304, 1907.



amounts (0.204 gram) of nitrogen. In this case no creatine appears in the urine. In my last paper on the subject<sup>1</sup> it is shown that the creatine induced by starvation may be made to disappear by administering calorically insufficient amounts of carbohydrates, while fats do not have this effect.

In the papers of Mendel and Rose above quoted it is shown that in the rabbit proteins do not have the effect of causing creatine which has been established by starvation to disappear from the urine. Relying on the evidence furnished by Cathcart for man that proteins do not prevent its appearance in carbohydrate hunger, they doubt the statement made by me at the December meeting of the Society of Biological Chemists in 1907 that pure protein feeding in the dog causes the disappearance of creatine from the urine after starvation. Apart from the fact that the actual results were at the disposal of the members of the society at that time, the recently published results indicate that the judgment of Mendel and Rose is at fault. It would seem to be unwise to draw inferences as to the metabolism of the dog from the behavior of man and the rabbit. We are already acquainted with a case, viz., acidosis, where the behavior of the dog is in no way comparable to that of man and the pig.

That protein, in the form of coagulated serum albumin washed free from any extractives, in an amount 39 per cent gross of the energy requirements—in reality, however, providing only 29 per cent of the caloric need—is sufficient to cause the disappearance of creatine from the urine is shown in my last paper beyond the limits of reasonable question. Only when a large quantity of meat was fed, in animal 381, were 64 mgms of creatine nitrogen excreted. With the considerable quantity of creatine-containing food ingested this was not an unexpected result.

That carbohydrate insufficiency is alone responsible for the appearance of creatine in the urine is rendered improbable by some of the results which were obtained in my former laboratory during the years I worked there at this problem.

In any discussion of creatine, it is most necessary, as it is in a discussion of acidosis, to be quite certain in decisive cases that the amount of food is well above the energy requirements of the body,

<sup>1</sup> Wolf *Biochem Zeitschr*, xxv, p 329, 1911

and that the amount of carbohydrate is sufficient Furthermore, we may take it as necessary that creatine-containing foods shall be excluded from the diet

In an investigation of day and night urines reported in this Journal,<sup>1</sup> a subject whose weight was constant throughout the experiment, and who partook mainly of cereal foods, excreted creatine throughout the period of examination

Other cases may be cited where the amount of food was more than sufficient to compensate for bodily loss under ordinary conditions, and where a large amount of carbohydrates was given, and yet creatine was found in the urine For example in case 18 of my paper with Ewing,<sup>2</sup> a patient weighing 71 kilos received arrow-root starch, rice butter and chicken broth (possibly not quite creatine-free) amounting to 1641 calories and still excreted considerable quantities of creatine in the urine This was undoubtedly a case of under-nutrition, but certainly not to the extent which occurred with the dogs mentioned in my last paper

Much more conclusive cases may be cited from the work of Lambert and myself<sup>3</sup> In case 12, a patient with pneumonia of a severe type, the patient received 2500 calories per day in the form of a creatine-free diet and excreted large quantities of creatine through the urine This case is compared in the same paper with a case of mild pneumonia where a patient receiving the same diet excreted no creatine in the urine The view is expressed in this paper that creatine may be excreted in severe cases of pneumonia, irrespective of the caloric value of the food ingested

This view is supported by the behavior of the second carbohydrate fed animal in my last paper This animal was obviously in a pathological condition, and died during the course of the experiment Although this animal received the same ration per kilo as the first, creatine was constantly present in the urine It would seem that the pathological condition of the animal, and not the presence or absence of carbohydrates in the food was responsible for the creatine excretion in this animal

One of the best examples of the independence of the creatine excretion of the carbohydrate metabolism is to be seen in the cases

<sup>1</sup> Osterberg and Wolf *This Journal*, III, p 165, 1907

<sup>2</sup> Ewing and Wolf *Archives of Internal Medicine*, IV, p 330, 1909

<sup>3</sup> Wolf and Lambert *Archives of Internal Medicine*, V, p 406, 1910

of rickets examined by Schwarz<sup>1</sup> in my laboratory. Rose finds, contrary to Schwarz, that children of five and over excrete creatine in the urine. The evidence produced by Rose is not conclusive, and for the following reasons. In the first place the term "perfectly normal" is one that cannot be accepted without further facts to show that such is really the case. Secondly, and more important, any results which are accompanied by the statement, "it is impossible to obtain information regarding the amount and kind of food eaten by the children" cannot be accepted without question. Obviously, in a situation where under-nutrition and the influence of creatine-containing foods may play so great a part, one cannot use results obtained from "city orphan homes or private families where the subjects of the experiments were ingesting more or less meat." An illuminating example of this type will be seen in the work of Williams and Wolf<sup>2</sup> on creatine excretion in cystinuria. In a series of analyses where no creatine was found, the subject "ate turkey" and immediately, 0.043 grams of creatine nitrogen appeared in the urine.

The data of Schwarz are of another character. Only one normal case was examined. The creatine-free food of the children was analyzed, and the resorption of protein, fat and carbohydrate estimated by an analysis of the feces. Not only this, but definite information is given to show that the children were in a positive nitrogen balance and gained weight throughout the experiment. In the cases of rickets examined, all excreted creatine, while the normal child was signalized by an absence of this compound from the urine. Between the completeness of the observations of Rose and of Schwarz there can be no choice. In the one case, the observer has acknowledged that he could obtain no information regarding the quantity and quality of the food, in the other all the data necessary for a consideration of the problem are available.

The statements of Amberg and Morrill<sup>3</sup> that sucklings excrete creatine in the urine are undoubtedly correct, but Rose omits to mention that these subjects received a diet containing both creatine and creatinine. The results cannot therefore be transferred to the question of whether older children on a creatine-free diet excrete this substance in the urine.

<sup>1</sup> Schwarz, *Jahrbuch für Kinderheilkunde*, lxxii, p. 549, 1910.

<sup>2</sup> Williams and Wolf, *This Journal*, vi, p. 337, 1909.

<sup>3</sup> Amberg and Morrill, *This Journal*, iii, p. 311, 1907.

Since coming to this laboratory, I have taken the opportunity to pursue my studies on rest nitrogen and creatine-creatinine excretion. Through the kindness of my colleague, Dr F Fischler, I have had placed at my disposal the large amount of material he possesses in Eck fistula dogs. These studies are naturally only in their beginning, but I am able to state that the changes which one observes in these animals are merely quantitative in type, not however for the reason which Mendel and Rose suggest. If these observers assume that the "hepatic function is entirely removed" they take a position, which, so far as I am aware, is not shared with them by any other physiologist today. As Fischler has pointed out, the arterial supply of this organ, after the vena portarum has been ligated, and connection between this vessel and the vena cava established, is quite sufficient to provide for all conditions of ordinary cell metabolism and nutrition. This is shown by the fact that the liver undergoes fatty degeneration as quickly and as completely in Eck animals as in the normal<sup>1</sup>. The quality and secretion of the bile is not markedly altered, and the tolerance for carbohydrates is apparently unchanged<sup>2</sup>. Hence the discussion of the results of London and Boljarski and of Foster and Fisher by Mendel and Rose loses some of its force.

Mendel and Rose have made an experiment with a phloridzinized dog to show the influence of glycogen upon creatine excretion. Their results are anomalous, and they state that no adequate explanation is apparent. They find that in a dog previously subjected to the influence of phloridzin the creatine disappears and reappears in an arbitrary way. In so far as I am aware, this experiment stands alone in the literature of creatine excretion in a fasting dog. My own experiments<sup>3</sup> seem to show that the creatine excretion during the course of fasting, and during the administration of phloridzin is much more regular in its behavior. In one of my own experiments where the animal died during the first four days of treatment with phloridzin, the creatine excretion outstripped the total nitrogen excretion, in that the creatine-total nitrogen ratio rose. Here it would seem that that creatine excretion was independent of the glyco-genic function and was influenced

<sup>1</sup> Fischler *Proc Naturforscher and Artze*, Karlsruhe 1911

<sup>2</sup> Fischler *Deutsch Arch f Klin Med*, civ, 1911

<sup>3</sup> Wolf and Osterberg *Amer Journ of Physiol*, xxviii, p 71, 1911

by other factors indicating a special pathological metabolic process

In view of the above considerations, I am led to believe that the explanation for the excretion of creatine in the urine will not be found to reside alone in a disturbance of the carbohydrate metabolism, nor in a disturbance of the liver, but that other processes are contributing to this end. In the course of the present winter, I hope to be able to show that this is the case.

# CREATINE AND CREATININE METABOLISM IN DOGS DURING FEEDING AND INANITION WITH ESPECIAL REFERENCE TO THE FUNCTION OF THE LIVER

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(Received for publication, November 27, 1911)

Certain authors have designated the liver as the organ chiefly concerned with the metabolism of creatinine. London and Boljarski<sup>1</sup> have given a very brief and unsatisfactory report upon the subject, in an article which appeared during the progress of our work, which was done during the winter of 1909-1910.

It seemed advisable, therefore, to ascertain what light an examination of the creatinine excretion in the Eck-fistula dog would throw upon the subject.

The object of these investigations was to determine the fate of creatinine and creatine when fed or subcutaneously introduced into normal and Eck-fistula dogs, on a creatine-free diet and during starvation.

The operation for the Eck-fistula was performed according to a modification<sup>2</sup> of Pawlow's operation.<sup>3</sup> As is well known the Eck-fistula consists, in brief, of an anastomosis of the portal vein with the inferior vena cava, and a ligation of the former at the liver hilus. Since the hepatic artery remains intact under these conditions, the liver tissue is supplied with arterial blood, but the blood from the spleen, pancreas, stomach and intestines which contains the products of splenic and digestive activity is not allowed to diffuse through the liver substance. It is carried

<sup>1</sup> E S London and N Boljarski *Zeitschr f physiol Chem*, lxi, p 467, 1909

<sup>2</sup> B M Bernheim, John Homans and Carl Voegtlin *Journ of Pharmacol and Exp Ther*, i, p 463 1910

<sup>3</sup> We are indebted to Dr Bernheim for performing the operations on the dogs that were used in these experiments

directly through the vena cava to the heart. Though the blood supply from the hepatic artery is relatively small, it appears to be adequate for keeping up the vitality of the liver cells.

Our intention was to determine the reaction of the same organism to the various conditions mentioned, but most unfortunately this was rendered impossible by the death of the animal before we had completed the observations. As the season, was, at that time, too far advanced to perform the complete experiment on another dog, we were compelled to make our observations on several animals.

The creatine used in these experiments was prepared from muscle and did not contain creatinine. The creatinine was obtained from human urine following the directions of Folin<sup>1</sup>. For the purification of this substance we found it advantageous to dissolve the creatinine in a small volume of warm 75 per cent alcohol and to precipitate this solution, after cooling, with ether. The first fractions thus obtained are absolutely free from creatine as far as could be determined by Folin's colorimetric method. This latter method was also used for the estimation of urinary preformed and total creatinine. Total nitrogen was determined by the Kjeldahl method. The urine was collected in 24-hour periods by catheterization.

<sup>1</sup> O. Folin *Zeitschr f physiol Chem*, xli, p 221, 1904

TABLE I

*Black and white terrier—Normal No 1*

Food 500 cc milk 300 cc water daily, given by stomach tube

DATE	URINARY OUTPUT	TOTAL CREATININE	PERFORMED CREATININE	CREATININE IN TUBES OF CREATININE	WEIGHT	NOTES
	cc	mg	mg			
January						
10	290	136	136	0		
11	300	141	141	0		
13	355	140	140	0		
14	380	167	167	0		
16	275	140	140	0		
17	330	140	140	0		
18	320	139	139	0		
19	270	138	138	0		
20	395	290	188	102		{ On January 20, at 4 p m , received by subcutaneous injection 60 cc of a creatine solution = 0.547 gram of creatine expressed as crea- tinine
21	370	204	166	38		
22	340	131	131	0		
23	360	138	138	0		
24	390	150	150	0		
25	450	303	168	135		{ On January 25, at 3 30 p m , received by subcutaneous injection 60 cc of the same creatine as before = 0.547 gram of creatine expressed as creatinine
26	280	107	107	0		
27	360	132	132	0		
28	320	102	102	0		
29	350	123	123	0		
30	410	138	138	0		
February						
3	365	169	169	0		
4	150	75	75	0	8,500	
5	335	124	124	0		
6	335	125	125	0		
7						
8	300	115	115	0		
9	300	115	115	0		
10	320	123	123	0		
11	325	133	121	12		{ On February 11, at 12 30 p m received by subcutaneous injection 50 cc of the same creatine solution = 0.456 gram creatine expressed as creatinine
12	260	83	83	0		
13	300	71	71	0		
14	260	83	83	0		
15	215	62	62	0		
16	275	129	129	0	7,200	



TABLE I—CONTINUED

DATE	URINARY OUTPUT	TOTAL CREATININE	PERFORMED CREATININE	CREATINE IN TERMS OF CREATININE	TOTAL NITROGEN	WEIGHT	NOTES
	cc	mgs	mgs		grams		
February							
17	220	111	111	0			
18	335	141	141	0			
19	365	118	118	0			
20	275	106	106	0			
21	325	116	116	0			
22	245	109	109	0			
23	300	121	121	0			
25	300	127	127	0	2	7,000	
26	360	278	145	133	2 1		February 26, 3 30 p m , received subcutan- ously 50 cc of crea- tine solution = 0 456 gram creatine ex- pressed as creatinine
27	210	124	124	0	1 1		
28	260	119	119	0	2 0		
March							
1	250	138	138	0	2 0		
2	290	93	93	0	2 0		
3	370	124	124	0	2 2		
4	290	103	103	0	2 0		
5	250	107	107	0	1 9		
6	325	94	94	0	1 9		
7	270	227	101	126	1 8		March 7 10 a m fed 50 cc creatine so- lution in milk = 0 - 456 gram creatine in terms of creatinine
8	270	128	128	0	2 1		
9	300	119	119	0	1 7		
10	380	205	205	0	1 9		March 10, fed 50 cc creatinine solution in milk = 0 275 gram creatinine
11	325	94	94	0	2 0		
12	300	273	240	33	2 4		March 12, subcutane- ous injection of 50 cc of same creatinine solution = 0 275 gram
13	285	138	85	53			
14	300	89	81	8			

*Experiment discontinued until April*

April							
3	650	144	144	0		9,100	
5	620	131	131	0			
5	610	124	124	0			
6	722	178	178	0	3 3		

TABLE I—CONTINUED

DATE	URINARY OUTPUT	TOTAL CREATININE	PER FORMED CREATININE	CREATININE IN TERMS OF CREATININE	TOTAL NITROGEN	WEIGHT	NOTES
	cc	mg	mg		grams		
April							
7	709	100	100	0	2 5		
8	627	150	150	0	3 2		
9	641	113	113	0	2 5		
10	638	137	137	0	2 0		
11	590	150	150	0		8,900	
13	720	102	102	0	1 1		
14	640	125	125	0	1 1		
15	535	113	113	0	1 3		
16	670	150	150	0	1 5		
17	680	158	158	0	3 9	8,500	
18	765	326	326	0	3 0		April 18, Subcutaneous injection of 0.5 gram of urinary creatinine solution, by colorimeter = 0.242 gram
19	575	111	111	0	2 4		
20	540	112	112	0	2 8		
21	680	328	283	45	3 2		April 21 Fed 0.272 gram of same creatinine as above
22	590	109	109	0	3 2		
23	400	135	135	0	1 4	8,500	
24	640	157	157	0	2 8		April 25 Fed 0.5 gram of creatine in 50 cc of water = 0.456 gram creatine in terms of creatinine
25	785	233	176	57	4 0		
26	530	112	96	16	2 1		
27	470	162	145	17	3 1		
28	590	86	86	0	1 8		
29	580	112	112	0	2 3	8,300	

TABLE II

*Ecl. Fistula made on April 30*

DATE	URINARY OUT-PUT	TOTAL CREATININE	PREFORMED CREATININE	CREATINE IN TERMS OF CREATININE	TOTAL NITROGEN	FOOD	WEIGHT
	cc	mgs	mgs	mgs	grams	cc	grams
May							
2	485	253	147	106	2 25	Milk, 900	7,750
3	470		164	none	2 25	Milk, 900	
4	460	139	108	31	2 22	Milk, 900	
5	335		115	none	3 17	Milk, 600	
6	230		92	none	3 40	Milk, 450	
7	140	87	76	11	2 10	Milk, 450	
8	250		95	none	2 90	Milk, 330	
9	170	78	66	12	1 83	Milk, 270	7,300
10	125		104	none	2 08	Water, 100 + milk, 400	
11	280		67	none	1 08	Water, 100 + milk, 400	
12	400	82	60	22	1 82	Water, 100 + milk, 400	
13*	430		57	19	2 67	Water, 100 + milk, 400	
14	280		53	none	1 54	Water, 100 + milk, 400	
15	400		68	none	1 56	Water, 100 + milk, 400	
16	190		42	none	1 16	Milk, 700	6,950
17†	320		95	25	1 58	Milk, 550	
18	429		77	17	1 83	Milk, 550	
19	440		76	none	1 88	Milk, 550	
20	340		65	none	1 06	Milk, 550	
21†	435	99	55	44	1 48	Milk, 550	6,920
22	380		75	none		Milk, 550	
23	470		74	none	1 57	Milk, 550	
24	440	104	49	55	1 87	Milk, 550	
25	350		85	none	1 90	Milk, 550	
26	430		81	none	1 98	Milk, 550	
27	390		69	none	1 79	Milk, 550, Sugar, 50 gms	
28	340		91	none	1 76	Milk, 550, 50 grams casein	6,350
29	400		98	none	2 00	Milk, 500, 50 grams casein	
30	345		84	none	2 22	Milk 50, 500, Water	

\* Fed 0.456 gram of creatine in terms of creatinine

† Subcutaneous injection of 0.456 gram of creatine in terms of creatinine

TABLE III

*Ecl Fistula Dog No 2*

Fed on 700 cc milk + 300 cc H<sub>2</sub>O + 10 grams of Ca-lactate daily (Same as starvation dog No 3 and 4)

DATE	URINARY OUTPUT	TOTAL CREATININE	PREFORMED CREATININE	CREATINE IN TERMS OF CREATININE	TOTAL NITROGEN	WEIGHT
	cc	mgs	mgs	mgs	grams	grams
June						
10	810	163	163	0	3 42	8,700
11	625	159	159	0	3 15	
12	700	159	159	0	3 15	
13	700	157	157	0	3 41	
14*	770	331	331	0	3 05	
15	600	174	174	0	2 88	
16	680	129	129	0	3 46	
17	745	120	120	0		
18	735	123	123	0		8,400
19†	760	303	303	0		
20	750	135	135	0		
21	650	131	131	0		
22	720	140	140	0		
23‡	740	164	164	0		
24	800	174	174	0	3 72	
25	820	141	141	0		
26	710	159	159	0	3 06	
27	720	135	135	0		
28	740	123	123	0	2 65	
29§	730	229	174	55	3 21	
30	650	169	155	14	2 45	
July						
1	565	123	123	0	2 91	
2	400	136	136	0		
3	720	171	171	0	1 74	
4**	740	503	503	0	2 73	8,000
5	775	172	172	0	2 77	
6	660	117	117	0	3 06	

\* Subcutaneous 50cc creatinine solution = 245 mgs creatinine

† Fed 50 cc creatinine solution = 245 mgs creatinine

‡ Fed 0.5 creatine = 350 mgs creatine in terms of creatinine

§ Subcutaneous 0.5 creatine = 350 mgs creatine in terms of creatinine

Fed 760 mgs of creatinine

TABLE IV  
*Starvation No I Later found to be pregnant*

DATE	URINARY OUT- PUT	TOTAL CREATININE	PREFORMED CREATININE	CREATINE IN TERMS OF CREATININE	TOTAL NITROGEN	FOOD	WEIGHT
	cc	mgs	mgs	mgs	grams		grams
April 24	210	57	47	10		300 cc water by stom- ach tube	9,340
25	280	98	87	11	2 21	300 cc water by stom- ach tube	
26	335	116	116	0	2 11	300 cc water by stom- ach tube	
27	250	76	76	0	2 59	300 cc water by stom- ach tube	
28	200	147	119	28	1 74	300 cc water by stom- ach tube	
29	270	161	119	42	2 41	300 cc water by stom- ach tube	
30	250	145	129	16	2 14	300 cc water by stom- ach tube	
May 1	280	177	177	0	2 97	300 cc water by stom- ach tube	7,900
2	210	179	135	44	2 19	300 cc water by stom- ach tube	
3	340	156	102	54	2 77	300 cc water by stom- ach tube	
4	210	170	106	64	2 46	300 cc water by stom- ach tube	
5	70	145	100	45	2 15	300 cc water by stom- ach tube	
6	150	173	126	47	2 97	300 cc water 60 grams cane sugar	7,650
7	185	174	111	63	2 35	300 cc water 60 grams cane sugar	
8	155	187	104	83	1 83	300 cc water 60 grams cane sugar	
9	140	169	82	87	2 24	300 cc water 60 grams cane sugar	
10	200	184	97	87	2 43	300 cc water 60 grams cane sugar	
11	155	189	93	96	2 15	+ 60 grams olive oil	
12	220	216	71	145	2 64	+ 60 grams olive oil	

TABLE V

*Starvation No II 500 cc water by stomach tube daily*

DATE	URINARY OUTPUT	TOTAL CREATININE	PREFORMED CREATININE	CREATINE IN TERMS OF CREATININE	TOTAL NITROGEN	WEIGHT
	cc	mg	mg	mg	grams	grams
February						
23	45	132	132	0		8,900
24	175	136	136	0	2 36	
25	265	144	144	0	1 99	
26	280	213	151	62	2 11	
27	160	221	164	57	2 99	
28	210	212	206	36	2 99	
March						
1	260	187	187	0	2 89	
2	120	216	186	30	2 73	
3	280	155	155	0	2 53	
4	250	168	168	0	1 8	
5	235	120	120	0	2 78	
6	280	185	185	0	3 58	
7*	235	243	184	59	3 50	
8	240	216	144	72	1 73	6,640
9	290	234	234	0	4 33	
10	235	154	154	0	2 84	
11	320	161	93	68	4 50	
12	270	187	89	98		5,900

\* Subcutaneous injection of creatine solution 50 cc = 0.456 gram in terms of creatinine

TABLE VI

*Starvation No III (Eck fistula made later same animal as Eck fistula dog fed No II and starved No IV (Eck fistula) 300 cc water daily*

DATE	URINARY OUTPUT	TOTAL CREATININE	PREFORMED CREATININE	CREATINE IN TERMS OF CREATININ	TOTAL NITROGEN	WEIGHT
	cc	mgs	mgs	mgs	grams	grams
March						
20	270	167	127	40	2 62	9,170
21	230	133	116	17	2 65	
22	270	151	151	0	3 19	
23	210	154	131	23	2 48	
24	240	172	140	32	2 61	
25	185	139	105	34	1 96	
26	190	158	102	56	2 01	7,820
27	230	196	124	72	2 47	
28	190	160	107	53	1 59	
29*	200	355	249	106	1 92	7,220
30	150	122	78	44	1 50	
31	235	72	72	0	2 17	
April						
1	260	66	66	0	1 82	
2	175	72	72	0	1 56	6,700
3†	300	154	128	26	2 16	
4	220	65	43	22	1 57	
5	180	55	55	0		

\* Subcutaneously 50 cc creatinine solution = 245 mgs creatinine

† Same amount as (\*) of creatinine by stomach tube

TABLE VII

*Starvation No IV (Eck Fistula) same as No II Eck fistula fed, and starvation No III, 500 cc water daily—stomach tube*

DATE	URINARY OUTPUT	TOTAL CREATININE	PREFORMED CREATININE	CREATINE IN TERMS OF CREATININE	TOTAL NITROGEN	WEIGHT
	cc	mgs	mgs	mgs	grams	grams
July						
7	450	146	146	0	2 08	8,000
8	470	133	131	2	2 93	
9	450	139	126	13		
12	350	180	124	56	3 33	7,000
13*	530	471	312	169	3 78	
14	500	225	106	119	3 61	
15	455	184	80	104	2 76	
16†	320	248	168	80	1 95	
17	490	197	96	101	2 52	
18	500	165	77	88	2 96	6,190

\* Subcutaneous injection of creatinine 50 cc 1 per cent solution = 245 mgs creatinine

† Same as (\*) given by stomach tube

SUMMARY OF EFFECTS OBSERVED ON THE EXCRETION OF URINARY  
CREATINE AND CREATININE AFTER THE ADMINISTRATION OF  
CREATINE AND CREATININE TO THE FED NORMAL AND THE FED  
ECK-FISTULA DOG

1 Creatinine excretion on a fixed diet is not invariable in amount  
a variation of 30 per cent may be expected in the normal animal

2 Creatine subcutaneously introduced into dogs may be followed by

a A slight increase in the creatinine excretion in the urine,  
(not invariable) as shown by the following results collected from  
Table I

January 20 Subcutaneously injected 0.547 mg creatine, 74 mg  
increase in creatinine

January 25 Subcutaneously injected 0.547 mg creatine, 28 mg  
increase in creatinine

February 11 Subcutaneously injected 0.456 mg creatine, 0 mg  
increase in creatinine

February 26 Subcutaneously injected 0.456 mg creatine, 15 mg  
increase in creatinine

No increase in creatinine was observed after feeding creatine

b The appearance of creatine in the urine (Table I)

January 20 Subcutaneously injected 0.547 gram creatine, 140 mgs  
creatine recovered

January 25 Subcutaneously injected 0.547 gram creatine, 135 mgs  
creatine recovered

February 26 Subcutaneously injected 0.456 gram creatine, 133 mgs  
creatine recovered

February 11 Subcutaneously injected 0.456 gram creatine, 12 mgs  
creatine recovered

March 7 Fed 0.456 gram creatine, 126 mgs creatine recovered

April 23 Subcutaneously injected 0.456 gram creatine, 90 mgs  
creatine recovered

3 Creatinine fed and introduced subcutaneously into normal  
dogs may be followed by

a The appearance of creatine in the urine (inconstant)  
Table I



March 10 Fed 275 grams creatinine, recovered 0 mgs creatine  
 March 12 Subcutaneously injected 275 grams creatine, recovered 94 mgs creatine  
 April 18 Subcutaneously injected 275 grams creatinine, recovered 0 mgs creatine  
 April 21 Fed 242 grams creatinine, recovered 45 mgs creatine

b Increase of creatinine in the urine—Table I

March 10 Fed 275 grams creatinine, increase of 100 mgs creatinine  
 April 21 Fed 272 grams creatinine, increase of 170 mgs creatinine  
 March 12 Subcutaneously injected 275 grams creatinine, increase of 150 mgs creatinine  
 April 18 Subcutaneously injected 242 grams creatinine, increase of 176 mgs creatinine

4 In Eck-fistula dog No 1 after operation a decrease of the creatinine excretion occurred which amounted to 29 per cent and was constant This result was not sustained by the findings in the second Eck-fistula dog of the same weight and upon the same diet

5 a Creatinine is excreted in smaller amounts both after feeding and after subcutaneous injection in the Eck-fistula dog than in the fed normal dog Eck-fistula dog No 1 Tables II and III

May 13 Fed 0 456 gram creatine, recovered 19 mgs (?) creatine  
 May 17 Subcutaneously injected 0 456 gram creatine, recovered 42 mgs creatine  
 May 21 Subcutaneously injected 0 456 gram creatine, recovered 44 mgs creatine

Eck-fistula dog No 2

June 23 Fed 0 380 gram creatine, Recovered 0 mgs creatine  
 June 29 Subcutaneously injected 0 380 gram creatine, Recovered 69 mgs creatine

b A slight increase occurs sometimes in the creatinine excretion after the administration of creatine to the fed Eck-fistula dog—Tables II and III

May 13 0 456 gram creatine fed, 0 mgs increase creatinine  
 June 23 0 380 gram creatine fed, 25 mgs increase creatinine  
 May 17 0 456 gram creatine subcutaneously injected, 39 mgs increase creatinine  
 May 21 0 456 gram creatine subcutaneously injected, 0 mgs increase creatinine  
 June 29 0 380 gram creatine subcutaneously injected, 50 mgs increase creatinine

6 In the three experiments in which creatinine was administered to Eck-fistula dog No 2 no creatine appeared after administration, though a large amount, 0.760 gram, was given (by mouth). The increase in the creatinine was as follows Table III

June 14 Subcutaneous injection of 0.245 gram creatinine, increase of 173 mgs creatinine

June 19 Fed 0.245 gram creatinine, increase of 180 mgs creatinine

July 4 Fed 0.760 gram creatinine, increase of 350 mgs creatinine

Seventy per cent of creatinine which was injected and 52 per cent of the fed creatinine was recovered. The increase of the urinary creatinine showed no difference in the fed Eck-fistula dog from the fed normal dog.

### *Starvation Experiments*

7 Creatine administered subcutaneously during starvation does not increase the creatinine excretion in the urine. The amount of creatine, which was recovered in the urine as creatine, was difficult to estimate, as creatine appeared irregularly during starvation. The recovered creatine seemed to be about 12 per cent of the amount given—Table V.

March 7 Subcutaneously injected 0.456 gram creatine, 59 mgs creatine recovered in urine.

8 Creatinine subcutaneously introduced into the starved dog gives rise to an increased creatine and creatinine excretion. When creatinine is fed much less of it is recovered—Table VI.

March 29 Subcutaneously injected 245 grams creatinine, recovered 53 mgs creatine + 142 mgs (58 per cent) creatinine.

April 3 Fed 245 mgs creatinine, recovered 20 (?) mgs creatine + 56 mgs (22 per cent) creatinine.

9 Creatinine if administered subcutaneously to the starved Eck-fistula dog causes an increase of the creatine and creatinine excretion in the urine, if fed no increase in the creatine and a much smaller increase in the creatinine occurs. This is the same result as was obtained in the starved dog—Table VII.

July 13 Subcutaneously injected 245 mgs creatinine, recovered 103 mgs creatine + 188 mgs (76 per cent) creatinine

July 16 Fed 245 mgs creatinine, recovered 0 grams creatine + 88 mgs (36 per cent) creatinine

### Discussion

About 25 per cent or less of the creatine introduced into normal fed dogs either by mouth or subcutaneously was recovered as creatine. This result may be explained by the relatively large amount used (Folin and other investigators, experimenting on man, administered smaller quantities of creatine per kilo of body weight. They were unable to recover creatine in the urine.) We therefore agree with Folin's interpretation, that *creatine must be considered as a food*<sup>1</sup>. Of the remaining 75 per cent of the creatine which does not reappear about 5 per cent is recovered occasionally as creatinine. This result is in accordance with the findings of v Hoogenhuyze and Verploegh<sup>2</sup> and Pekelharing and v Hoogenhuyze<sup>3</sup>. However, the increase in the creatinine after creatine administration is not constant, nor, when it occurs, does it exceed greatly the normal variation of the creatinine excretion. When creatine is introduced *per os* or subcutaneously into the Eck-fistula dog less creatine (9 per cent is recovered than in the normal dog (23.5 per cent). As to the effect of the administration of creatine on the creatinine excretion of the Eck-fistula dog no difference from the effect on the normal dog was observed.

*The Eck-fistula dog reacts to the administration of creatine in exactly the same way as the normal dog.* The difference in the actual amounts of the recovered creatine is too small to allow conclusions to be drawn from it. It was our experience with a large number of Eck-fistula dogs that, unless on a special diet, they lost weight. The small amounts of creatine which appear at times in the urine of Eck-fistula dogs (see table II, Eck-fistula dog No I and Foster and Fisher<sup>4</sup>), may be well accounted for by the insuffi-

<sup>1</sup> O. Folin *Festschrift für Hammarsten*, 1906

<sup>2</sup> van Hoogenhuyze and Verploegh *Zeitschr f physiol Chem*, LVII, p 131, 1908

<sup>3</sup> C. A. Pekelharing and C. T. C. van Hoogenhuyze *Zeitschr f physiol Chem*, LVII, p 407, 1910

<sup>4</sup> N. B. Foster and H. L. Fisher *this Journal*, LV, p 559, 1911

cient diet they received and the concomitant loss of weight. The condition, therefore, may be regarded as one of partial starvation and the appearance of the creatine may be explained in the same manner as its presence during inanition.

About 57 per cent of the creatinine administered to the fed normal dog is excreted as creatinine and 43 per cent does not appear as creatinine. The appearance of creatine after the administration of creatinine occurred twice in the normal dog in small amounts.

Creatinine administered to the fed Eck-fistula dog is recovered in about the same amounts as when given to the fed normal dog. We infer from this that the *exclusion of the portal circulation from the liver has no effect upon the creatinine metabolism*.

In starvation the daily variation of the creatine as well as the creatinine is very great, especially as starvation progresses. Having observed in starvation dog No. 1 that the creatinine excretion remained fairly constant for a considerable period, we confined most of our experiments to the introduction of creatinine.

Creatinine when administered both by mouth and subcutaneously during inanition is recovered in much smaller amounts than in the case of the fed dog. The amount recovered is smaller when the creatinine is fed (22 per cent), than when it is injected subcutaneously (58 per cent). The same diminution of recovered creatinine after creatinine administration is observed in the starved Eck-fistula dog though to a less extent.

The recovery of small amounts of creatinine in the case of the starved dog is in complete accordance with the administration of creatine to the fed dog. In the latter case 24 per cent of the amount of creatine introduced was recovered. *Creatinine may be used by the starving organism as food in the same way in which we suppose that the well nourished organism uses the creatine*.

The fact that creatine and creatinine are excreted during starvation does not contradict this view. Starvation is a more or less pathological condition of metabolism, this is shown by the excretion of abnormal unoxidized substances, such as oxybutyric, diacetic, lactic acids. It may be well assumed that, as Pekelharing and v. Hoogenhuyze have suggested, enzymatic actions are diminished during inanition. On the other hand muscle protein is broken up in considerable amounts during inanition, and as is known, this results in liberation of relatively great quantities of

creatinine which may be excreted before destruction occurs. It is difficult to decide which of these views is correct. Perhaps both processes play a part.

This result of creatinine administration in the starved dog emphasizes the effect of creatinine administration to the fed dog. In the latter case 25 per cent or 30 per cent does not reappear. The fact, that ordinarily creatinine is very readily excreted by the kidney, may account for its presence in the urine and that portion of it, which appears may be regarded as an end product of protein metabolism, since it is not destroyed. However a certain portion of the creatinine introduced into fed animals and a greater portion of creatinine introduced into starved ones, does not reappear. This is sufficient evidence that the substance is not a final end product, but may be further broken down. Since this is shown to be the case experimentally, we have reason to suppose that in the body there is normally a physiological use for unexcreted creatinine. We may regard the process of creatine and creatinine metabolism as dependent upon three factors: Production, Destruction and Excretion.

We gave the whole amount of creatine or creatinine at once, the most unfavorable method for destruction, given at intervals through the twenty-four hours, as was done by Pekelharing and van Hoogenhuyze, a much greater percentage of the amount administered failed to reappear. Similarly a much larger amount was recovered by the above mentioned authors, when they introduced the substance intravenously, than when they gave it subcutaneously, and by us when we gave it subcutaneously, than when we gave it by mouth. The difference was evidently due to the length of time the substance was exposed to the action of the tissues.

*The results of our work tend to show that the liver is not an organ of prime importance in connection with creatine and creatinine metabolism.* We found no marked difference in the behavior of normal and Eck-fistula dogs to creatine and creatinine. This is in complete accordance with the work of Foster and Fisher<sup>1</sup> on the same subject. Our experiments do not corroborate the hypothesis advanced by Mellanby<sup>2</sup>

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<sup>1</sup> N. B. Foster and H. L. Fisher, *this Journal*, ix, p. 559, 1911.

<sup>2</sup> E. Mellanby, *Journ. of Physiol.*, xxxi, p. 447, 1908.

"As regards the action of the liver I suggest that it is continuously forming creatinine from substances carried to it by the blood stream from other organs and that in the developing muscle this creatinine is changed to creatine and stored, while after the muscle has reached saturation point, creatinine is continuously excreted" (page 486)

Speaking of the creatinine formation in developing chicks this author says

"The increase in the rate of growth of the liver at hatching is no doubt due to the closing of the ductus venosus so that a much greater bloodsupply reaches this organ. The fact that the growth of the liver goes on at the same rate as that of the rest of the chicken during most of the incubation period, would seem to show that during this time its special functions are not particularly called upon. Were this not the case, the ductus venosus would, no doubt, close sooner. The first function of any organ is to grow and the increased bloodsupply to the liver at hatching causes the corresponding increase in the rate of growth of this organ. This closing of the ductus venosus is a very special change, and it is not likely that there are many similar changes in the body at this time, which do not depend on the increased bloodsupply to the liver. It is therefore reasonable to put forward the synchronous rates of growth of the liver and creatine both during incubation and after hatching as strong evidence of an intimate connection between the bloodsupply of the organ and the development of creatine. The many functions of the liver would make one hesitate to conclude that this connection was direct—that, in fact, creatine was made in the liver and stored as such in the muscle" (page 477)

From this we see, that Mellanby regards the increased blood flow through the liver as the important factor in creatine formation. It is hardly conceivable that the liver could double its weight on the day of hatching by an increase of cellular tissue (figures quoted by Mellanby), such an increase in weight must be due to engorgement with blood. This is a probable occurrence as it is known that after the closure of the ductus venosus more blood is carried to the liver by the portal system. The opposite of this condition of liver engorgement is found in the Eck-fistula dog where the bloodsupply of the organ is reduced to a minimum by the exclusion of the portal circulation. If Mellanby's hypothesis were correct, we would expect to find a very great reduction in the creatinine excretion or an absence of creatinine in the urine of an Eck-fistula dog, but as this does not occur we must consider that our results show his hypothesis to be an erroneous one. Amberg and

Rowntree<sup>1</sup> have already arrived at the same conclusion by an indirect mode of approach. We do not deny, of course, the possibility of creatine formation through catabolic processes in the liver, as in other tissue cells. By perfusion experiments Gotthieb and Stangassinger<sup>2</sup> have shown for the liver, and Howell and Duke<sup>3</sup> and Weber<sup>4</sup> for the contracting heart, that creatine is found in the perfused fluid. Creatine is also formed during autolysis by various organs.

In our experiments the diminution of the liver function was studied under conditions which we believe to be as nearly physiological as can be attained. That there is a true reduction of the liver function in Eck-fistula dogs has been shown by Pawlow and Nencki<sup>5</sup> in the case of urea synthesis from ammonium salts and by Voegtlin and Bernheim<sup>6</sup> in the case of bile formation.

Those authors who have attempted to determine the cause of creatinine metabolism in an organism in which the hepatic function was impaired, have used for their observations patients in an advanced stage of supposed hepatic insufficiency, caused by cirrhosis or carcinoma of the liver or eclampsia, or they have chosen animals poisoned by hydrazine, etc. Under these conditions many other abnormalities, beside the reduction of the function of the liver must be present, such as the toxic destruction of tissues in general and also starvation due to the refusal of food during the intoxication.

#### CONCLUSIONS

1 The more or less constant excretion of creatinine in an animal on a fixed diet is explained by the constancy of the three factors which determine it, viz, *production* in the course of catabolism, *destruction* through the action of enzymes, and *kidney secretion*.

2 In the dog creatinine and creatine are not true end products.

<sup>1</sup> S. Amberg and L. G. Rowntree *Johns Hopkins Hospital Bull.*, xvi, No 227, 1910.

<sup>2</sup> R. Gotthieb and R. Stangassinger *Zeitschr. f. physiol. Chem.*, lii, p. 1, iv, p. 322. R. Stangassinger *Zeitschr. f. physiol. Chem.*, lvii, p. 131, 1908.

<sup>3</sup> Howell and Duke *Amer. Journ. of Physiol.*, xiiii, p. 174, 1908.

<sup>4</sup> Weber *Arch. f. exp. Path. u. Pharm.*, lviii, p. 93, 1907.

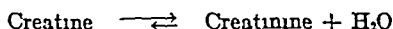
<sup>5</sup> Hahn, Massen, Nencki and Pawlow *Arch. f. exp. Path. u. Pharm.*, xxvii, p. 161, 1893.

<sup>6</sup> Voegtlin and Bernheim *Journ. of Pharm. and Exp. Ther.*, ii, p. 455, 1911.

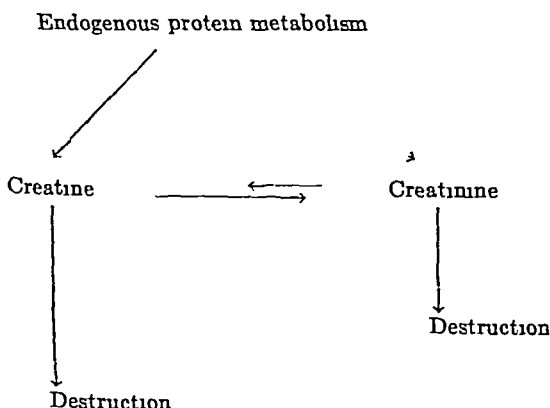
of metabolism That portion of these substances which appears in the urine is due to the fact that it is excreted by the kidney before destruction occurs

3 It is shown that the liver does not play the important rôle in reference to the creatine metabolism that has been ascribed to this organ

4 The occasional appearance of creatine in the urine after creatinine administration as well as the occasional increase of urinary creatinine after creatine administration, suggests the possibility of creatinine hydrolysis being a reversible reaction in the animal organism



5 Our present views on creatine and creatinine metabolism in general may be expressed by the following scheme (The length of the arrows indicates the extent of the reaction and the dotted arrow indicates that the origin of creatinine directly in the course of endogenous metabolism is doubtful)







# THE CATABOLISM OF HISTIDINE

BY H D DAKIN AND A J WAKEMAN

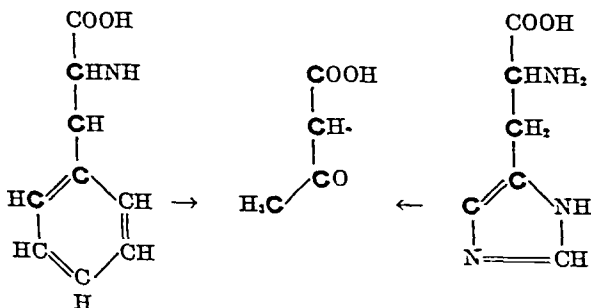
(From the Herter Laboratory, 819 Madison Avenue, New York )

(Received for publication, December 4, 1911 )

In a recent issue of this Journal the authors brought forward evidence concerning the mechanism of the formation of acetoacetic acid from phenylalanine and from tyrosine<sup>1</sup> From their experiments it appeared that the acetoacetic acid molecule was derived from four adjacent carbon atoms, two of them situated in the side-chain and two in the nucleus Homogentisic acid was believed not to be a normal intermediate product of the reaction

On considering the nature of this curious transformation it was realized that the histidine molecule contained four carbon atoms, two in the nucleus and two in the side-chain similarly related those which go to form acetoacetic acid in the phenylalanine molecule It appeared not improbable therefore that histidine might undergo a reaction in the body with formation of acetoacetic acid similar in type to that experienced by the aromatic amino-acids

The structural relation of these substances is readily seen from the following formulae



<sup>1</sup> This Journal, ix p 139, 1911

With the idea of testing the hypothesis we have made a number of experiments upon the perfusion of dogs' livers with a mixture of dog's and bullock's blood to which histidine carbonate had been added. The technique of these experiments was similar in every way to those previously reported<sup>1</sup> and after a perfusion lasting usually about fifty minutes the blood was analyzed for acetone and acetoacetic acid in the usual fashion.

The results are not as decisive as those obtained by Embden and others with tyrosine or phenylalanine but they do appear to show that a slight increase in the acetoacetic acid of the blood may follow addition of histidine carbonate. The results resemble those obtained by Embden with *laevo*-leucine. In the case of this amino-acid although the inactive synthetic compound readily gives much acetoacetic acid, the naturally occurring form gives it much less readily.<sup>2</sup>

Most of the histidine preparations employed by us were obtained by Fraenkel's method through the hydrolysis of blood. The pure crystalline hydrochloride was dissolved in water and decomposed by an equivalent addition of sodium bicarbonate. In one experiment the histidine solution was obtained by precipitation with phosphotungstic acid and subsequent saturation of the solution of the base with carbon dioxide. No difference in the results was observed.

The results are comprised in the following table

NUMBER	WEIGHT OF DOG	VOLUME PERFUSION FLUID	TIME	HISTIDINE ADDED	ACETO- ACETIC ACID	AVERAGE
	kilos	cc	minutes	gm	mg	
I	8	1,025	50	2.5	94	86
II	11	1,200	50	2.5	66	
III	11	1,200	50	2.5	99	
IV	13	1,000	50	2.0	76	
V	13	1,300	40	2.5	74	
VI	20	1,500	50	3.0	107	
VII	7.5	1,000	70		46	54
VIII	9	1,000	50		66	
IX	10	1,000	55		47	
X	10	1,200	50		48	
XI	21	1,300	45		62	

<sup>1</sup> *Loc cit*

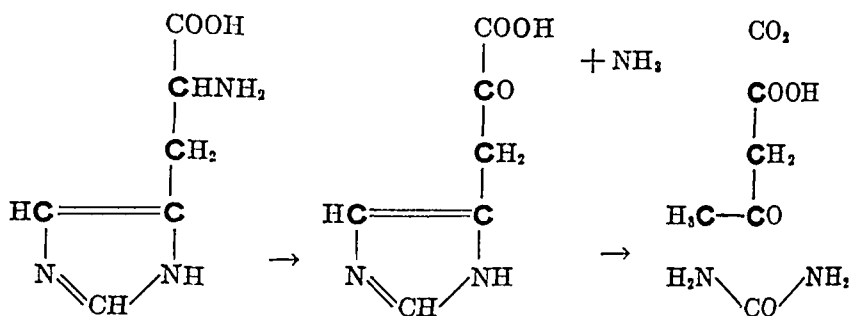
<sup>2</sup> Embden *Beitr z chem Physiol u Path*, vi, p 348, 1903

The ether used as anaesthetic during the operative part of the experiment was specially purified by repeated shaking with iodine and caustic soda solution and special blank determinations on the blood drawn at the close of the operation showed that no appreciable amount of acetone had been introduced

The animals employed varied considerably in size but blank experiments on animals of equal weight were performed The animals were allowed to fast for twenty-four hours previous to operation<sup>1</sup>

Inspection of the figures contained in the table shows that on the average an increase in acetoacetic acid corresponding to about 60 per cent over the normal blank perfusion experiments follows addition of histidine carbonate to the blood used for perfusion It is unfortunate that the increase is not more marked so that the results might be more definitely interpreted But we think it may be tentatively concluded that there is some evidence of acetoacetic acid formation from histidine Judging by analogy with the reactions observed in the oxidation of other amino-acids (Neubauer) it appears likely that the alanine side-chain of the histidine molecule is converted into an acetic acid group by intermediate formation of the corresponding ketonic acid, iminazolpyruvic acid, and liberation of ammonia The formation of acetoacetic acid necessitates the disruption of the ring It may be noted that while the formation of the ketonic acid from histidine involves oxidation, its subsequent decomposition to form urea and acetoacetic acid only necessitates oxidation in the side-chain The resolution of the nucleus is represented as simply requiring intramolecular rearrangement and the taking up of two molecules of water The N-CH-NH group would undoubtedly form urea The changes may be represented as follows

<sup>1</sup> The writers observed a markedly increased acetoacetic acid formation on perfusing the liver of a dog which had received much very fat meat some twelve hours before operation The blood serum was full of suspended fat particles We propose to investigate this phenomenon



The net result of the series of changes pictured above shows the histidine molecule resolved into carbonate of ammonia, which may then form urea, acetoacetic acid, which may then undergo further oxidation reduction or hydrolysis, and a urea group derived from the nitrogen atoms in the ring

Such a series of changes would accord admirably with the experiments of Abderhalden and Einbeck<sup>1</sup> and Kowalewsky<sup>2</sup> who found histidine when given to dogs to be almost completely decomposed apparently with the excretion of no other products of catabolism other than urea

We realize that additional experimental work is much needed in order to test the correctness of our tentative hypothesis as to the mechanism of histidine catabolism. We are at present engaged in the investigation of the fate of histidine in the diabetic organism, and by means of these and other experiments we hope to obtain some further insight into the mechanism of the reactions

<sup>1</sup> *Zeitschr f physiol Chem*, lvi, p 322, 1909, lxxviii, p 395, 1910

<sup>2</sup> *Biochem Zeitschr*, xliii, p 1, 1910

#### ERRATUM

On page 322 of this volume, No 4, first line of text, for *glutennn* read *gladin*

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